

UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA



Evaluation of antiviral drugs against BK polyomavirus

Carolina Martins da Conceição

Mestrado Integrado em Ciências Farmacêuticas

2019

UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA



Evaluation of antiviral drugs against BK polyomavirus

Carolina Martins da Conceição

Trabalho de Campo de Mestrado Integrado em Ciências Farmacêuticas apresentado
à Universidade de Lisboa através da Faculdade de Farmácia

Orientador: Doutor Gilles Duverlie

Co-orientador: Doutor José Miguel Azevedo Pereira, Professor Auxiliar com Agregação

2019

This project was developed under the Erasmus+ Programme, at the AGIR (AGents Infectieux, Résistance et chimiothérapie) unit, CURS (Centre Universitaire de Recherche en Santé), belonging to Université de Picardie Jules Verne in Amiens, France, under the supervision of Professor Gilles Duverlie.



Abstract

BK polyomavirus (BKPyV) is a DNA virus belonging to the *Polyomaviridae* family. BKPyV is considered ubiquitous among the population, and primary infection usually occurs during early childhood. This primary infection is usually asymptomatic, following which the virus establishes a lifelong persistent infection mainly on epithelial cells of the urinary tract and the kidney. In immunocompetent individuals the virus remains clinically silent throughout the life of the host. Immunosuppression, however, can lead to reactivation of the latent infection. In such conditions, one of the major clinical complications of BKPyV reactivation is polyomavirus-associated nephropathy (PyVAN) in kidney transplant recipients. The development of more potent immunosuppressant drugs has increased the incidence rate of PyVAN in these patients and to date, because a specific and safe antiviral drug against BKPyV is still lacking, the mainstay approach to avoid PyVAN development and the possibility of graft loss is to reduce the immunosuppressive treatment. In this context, the development and search for antiviral drugs that can efficiently stop BKPyV replication has become of great clinical interest.

This project focused on studying the efficacy of two drugs - NA-17 and TN-18 - against BKPyV replication in hTERT immortalized human renal proximal tubular epithelial cells (RPTEC/TERT1), an immortalized cell line of the major target cells of the virus *in vivo*. Results showed an inhibition of BKPyV infection with both NA-17 and TN-18, having been estimated effective concentration 50% (EC₅₀) values of 190 µM and 682 µM, respectively. Treatment of BKPyV-infected RPTEC/TERT1 with the highest concentrations tested for both drugs was responsible for a decrease of the extracellular BKPyV DNA load, that although discrete, corresponded also with an inhibition of the release of infectious progeny. Overall our results show a moderate antiviral effect against BKPyV for both drugs, making us believe that they would have only a limited effect on treating this infection *in vivo*. Further research in this area is needed to develop an efficient treatment against BKPyV infection and to improve disease outcomes in kidney transplant recipients that develop PyVAN as a result of BKPyV infection reactivation.

Keywords: polyomavirus, BK polyomavirus, antiviral, nephropathy, kidney transplantation

Resumo

O poliomavírus BK é um vírus de DNA pertencente à família *Polyomaviridae*. A infecção primária é normalmente assintomática, após a qual o vírus estabelece uma infecção persistente maioritariamente nas células epiteliais do trato urinário e do rim. Em indivíduos imunocompetentes o vírus permanece clinicamente silencioso durante toda a vida do hospedeiro. No entanto, a imunossupressão pode conduzir à reativação do vírus latente, sendo uma das principais complicações desta reativação a nefropatia associada a poliomavírus em transplantados renais. O desenvolvimento de imunossupressores cada vez mais potentes tem aumentado a incidência da nefropatia nestes doentes e atualmente, não existindo nenhum antiviral que possa ser utilizado de forma eficaz e segura contra este vírus, a abordagem clínica consiste na redução do tratamento imunossupressor de modo a prevenir o desenvolvimento da nefropatia. Neste contexto, o desenvolvimento e a procura de antivirais que consigam eficazmente impedir a replicação do poliomavírus BK têm assumido um grande interesse clínico.

O principal objetivo deste projeto consistiu no estudo da eficácia de dois compostos - NA-17 e TN-18 - contra a replicação do poliomavírus BK em células epiteliais tubulares proximais renais imortalizadas com hTERT (RPTEC/TERT1), uma linha celular imortalizada de um dos maiores alvos celulares do vírus *in vivo*. Os resultados mostraram uma inibição da infecção pelo poliomavírus BK, tendo sido estimadas as concentrações responsáveis por uma inibição de 50% da infecção de 190 μ M e 682 μ M para o NA-17 e o TN-18, respetivamente. O tratamento com as concentrações mais elevadas testadas para ambos os compostos foi responsável por uma redução da carga extracelular de DNA viral, assim como na redução de libertação de novas partículas virais infecciosas. De um modo geral, os resultados mostram um efeito antiviral moderado de ambos os compostos contra o poliomavírus BK, mostrando que teriam um efeito limitado no tratamento desta infecção *in vivo*. A continuação da investigação nesta área é necessária para o desenvolvimento de um tratamento eficaz da infecção pelo poliomavírus BK e para a melhoria do prognóstico nos transplantados renais que desenvolvem nefropatia como resultado da reativação desta infecção.

Palavras-chave: poliomavírus, poliomavírus BK, antiviral, nefropatia, transplante renal

Acknowledgments

First, I would like to thank Professor Gilles Duverlie, supervisor of this work. I am sincerely grateful to you for accepting me in your lab to develop this project. Thank you for sharing with me your knowledge and for guiding me through all this work.

To Professor José Miguel Azevedo Pereira for the co-supervision of this work. Thank you for all the support and words of encouragement even at a distance, and the availability on discussing the results and revising this thesis. Your contribution was essential to this work, and for that I am very thankful.

A very special thanks to all the team from the Virology lab at Amiens. To Veronique Descamps and Lynda Handala for sharing with me your experience, and above all for all the time and patience you had to teach me and answer all my questions. Also, to Fatima Dakroub, Tony Fiore, François Helle, Etienne Brochot, François Peltier, Virginie Morel, Catherine François, Baptiste Demey, Laurence Marquis and Catherine Moriset, thank you all for the way you welcomed me in Amiens and in your team, it was a pleasure having spent these three months with you. Merci à vous!

Also, I would like to thank Marta Calado. It was two years ago that I took my first steps on a research lab with you. All you taught me these years was fundamental to get me here.

To my friends that shared with me these last five years, Catarina, Inês, Cátia, Marta, Xana, Cláudia and Sofia. Thank you so much for your friendship, for your patience, and above all, for never letting me give up. Also, to Ana, Henrique and Inês, thank you so much for always being there for me.

To my mother and my sister, thank you for always having my back. Thank you for your unconditional support through all my worries and insecurities. I couldn't have done this without you.

List of abbreviations

BKPyV	BK polyomavirus
c/mL	copies/mL
CC₅₀	cytotoxic concentration 50%
CC₉₀	cytotoxic concentration 90%
CI	confidence intervals
CSK	cytoskeletal buffer
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
dpi	days post-infection
hpi	hours post-infection
dsDNA	double-stranded DNA
EC₅₀	effective concentration 50%
EC₉₀	effective concentration 90%
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FBS	fetal bovine serum
FAM	6-carboxyfluorescein
GA-100	gentamicin and amphotericin B
HEK293	human embryonic kidney 293
HLA	human leukocyte antigen
hTERT	human telomerase reverse transcriptase
IARC	International Agency for Research on Cancer
IVIG	intravenous immunoglobulin

JCPyV	JC polyomavirus
kbp	kilobase pairs
MCPyV	Merkell cell polyomavirus
miRNA	microRNA
MOI	multiplicity of infection
MPyV	mouse polyomavirus
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NAb	neutralizing antibodies
NCCR	non-coding control region
NLS	nuclear localization sequences
ORI	origin of replication
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pre-miRNA	precursor-miRNA
PyVAN	polyomavirus-associated nephropathy
qPCR	quantitative real-time PCR
REGM	renal epithelial growth medium
RPTEC	human renal proximal tubular epithelial cells
RPTEC/TERT1	hTERT immortalized RPTEC
RT-qPCR	reverse transcription qPCR
SI	selectivity index
SV40	simian virus 40

TAg	large tumor antigen
tAg	small tumor antigen
TCID₅₀	50% tissue culture infectious dose
truncTAg	truncated TAg
TSPyV	Trichodysplasia spinulosa-associated polyomavirus
VLP	virus-like particle

Table of Contents

Abstract.....	3
Resumo.....	4
Acknowledgments	5
List of abbreviations	6
Table of Contents.....	9
List of Figures.....	10
List of Tables.....	10
1. Introduction	11
1.1. BK polyomavirus	11
1.1.1. BKPyV virion	12
1.1.2. BKPyV genome.....	13
1.1.2.1. Genotypes and variants	14
1.1.3. BKPyV replication cycle	15
1.2. Primary infection, latency and reactivation	17
1.3. Polyomavirus-associated nephropathy	18
1.3.1. Early screening and diagnosis.....	19
1.3.2. Therapeutic approaches.....	20
1.4. Model systems for the study of BKPyV infection.....	24
2. Aims of this study	26
3. Materials and Methods	27
3.1. Cell culture	27
3.2. Virus.....	27
3.2.1. Virus stock production	27
3.2.1. Virus stock titration.....	27
3.3. Infection and drug treatment.....	28
3.4. Immunofluorescence staining, microscopy, and digital image processing	28
3.5. Extracellular BKPyV DNA load	29

3.6. Release of infectious progeny	30
3.7. Calculation of the effective concentrations 50%	30
4. Results	31
4.1. Effect of NA-17 and TN-18 on BKPyV infection on Vero cells.....	31
4.1.1. Inhibition of BKPyV infection	31
4.2. Effect of NA-17 and TN-18 on BKPyV infection on RPTEC/TERT1	32
4.2.1. Inhibition of BKPyV infection	32
4.2.2. Effect on BKPyV genome replication.....	34
4.2.3. Effect on the release of infectious progeny.....	35
5. Discussion.....	37
6. Conclusions and Future Outlooks.....	41
7. References.....	44

List of Figures

Figure 1. Cryo-electron microscopy structure of BKPyV virions.	12
Figure 2. Structure of BKPyV genome.....	13
Figure 3. Schematic representation of BKPyV replication cycle.....	15
Figure 4. Description of the QuantIF macro.....	29
Figure 5. Effect of increasing concentrations of NA-17 and TN-18 on the inhibition of BKPyV infection on Vero cells.	32
Figure 6. Effect of increasing concentrations of NA-17 and TN-18 on the inhibition of BKPyV infection on RPTEC/TERT1.....	33
Figure 7. Effect of increasing concentrations of NA-17 and TN-18 on BKPyV DNA load. ...	34
Figure 8. Effect of increasing concentrations of NA-17 and TN-18 on the release of infectious progeny.....	35

List of Tables

Table 1. Calculated EC ₅₀ values for NA-17 and TN-18 tested on RPTEC/TERT1.....	34
--	----

1. Introduction

1.1. BK polyomavirus

BK polyomavirus (BKPyV) is a member of the *Polyomaviridae* family of non-enveloped, small viruses, with 40 to 45 nm in diameter, and circular double-stranded DNA (dsDNA) genomes comprising approximately 5 kilobase pairs (kbp) (1). The polyomavirus family was first named after its founding member, the mouse polyomavirus (MPyV), which was found to cause tumors in mice (polyoma meaning “many tumors”) (2). This family has now more than eighty different viruses divided in four genera, each of them having a specific host range, including mammals, birds and fish (1).

Polyomaviruses with the ability to infect humans were first described in 1971. BKPyV was first isolated by inoculating Vero cells with urine samples of a patient who exhibited chronic pyelonephritis and advanced renal failure, and that for those reasons had undergone kidney transplant (3). In the same issue of *The Lancet*, independently, it was reported the isolation of JC polyomavirus (JCPyV) from brain tissue of a patient with Hodgkin's disease who had developed progressive multifocal leukoencephalopathy (4). These viruses, both named after the patients' initials, were the first two human polyomaviruses to be isolated. After these, numerous human polyomaviruses have been described, and to date, there are 13 polyomaviruses that are known to infect humans (1).

Human polyomaviruses are considered ubiquitous among the population, but primary infections with these viruses are generally not associated with significant clinical symptoms or disease. Immunosuppression, however, can lead to reactivation of the latent infection, and in such conditions there are four different human polyomaviruses that have been associated with human diseases (1). BKPyV is one of these viruses, and major complications, when reactivated, include polyomavirus-associated nephropathy (PyVAN) and hemorrhagic cystitis in kidney and hematopoietic stem cell transplant recipients, respectively (5). Additionally, JCPyV is associated with progressive multifocal leukoencephalopathy (6), Trichodysplasia spinulosa-associated polyomavirus (TSPyV) with trichodysplasia spinulosa (7), and Merkel cell polyomavirus (MCPyV) with Merkel cell carcinoma (8). Concerning their oncogenic potential, current evidence only supports the role of MCPyV as carcinogenic to humans, being classified by the International Agency for Research on Cancer (IARC) as “probably carcinogenic to humans” (group 2A). Other human polyomaviruses have already been found in tumors and efforts have been made to clarify the link between infection with these viruses and tumor development. However, their role in human cancer is not completely understood and BKPyV and JCPyV are only classified as “possibly carcinogenic to humans” (group 2B)

(9,10). As for the other human polyomaviruses, a relation between infection and disease development as not yet been established (10).

BK virus shares approximately 75% genome homology with JCpV and also 70% homology with simian virus 40 (SV40) (11), a virus first isolated from rhesus monkey kidney cells that had been used for poliovirus vaccine production (12).

1.1.1. BKPyV virion

BKPyV virions are non-enveloped, with approximately 45 nm in diameter. The viral capsid, with an icosahedral symmetry, is composed of three structural proteins, the major capsid protein VP1, and the two minor capsid proteins VP2 and VP3. On the outer surface of the capsid, 360 copies of VP1 form 72 pentamers arranged in a T=7d icosahedral structure (13) (Figure 1). The pentamers are linked between them through the C-terminal arm of VP1, and are further stabilized by disulfide bonds and calcium cations (14). Each of these pentamers is associated with a single molecule of either VP2 or VP3 on the inner surface of the capsid (13).

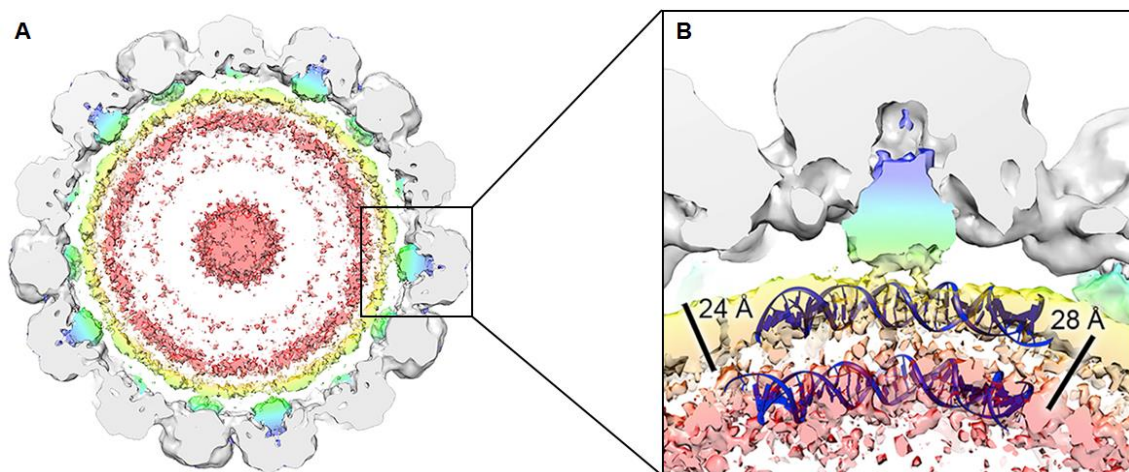


Figure 1. Cryo-electron microscopy structure of BKPyV virions. (A) View of a 40-Å thick slab through the unsharpened/unmasked virion map shown at a contour level of 0.0034. Pyramidal density below each VP1 penton and two shells of electron density adjacent to the inner capsid layer can be seen. The density within 6 Å of the fitted coordinates for SV40 VP1 is colored gray. The remaining density is colored in a radial color scheme. Densities for VP2 and VP3 are colored blue and green, and for packaged dsDNA yellow and pink. (B) Enlarged view of the pyramidal density beneath a single VP1 penton of the virion shown at a contour level of 0.0032. Strands of dsDNA wrapped around a human histone octamer (PDB: 1AOI) are shown, indicating that the two shells of density have a comparable spacing. Discrete connective density between the pyramidal density and internal shells is also apparent. (Adapted from Hurdiss, et al., 2016)

The capsid encloses the viral genome, a single circular dsDNA molecule, packed with the cellular histones H2A, H2B, H3 and H4 under the form of a minichromosome containing around 20 nucleosomes (13).

1.1.2. BKPyV genome

The BKPyV genome comprises about 5 kbp and it is divided into two transcriptional regions, the early and the late coding regions (referring to the phase of the infection in which each of them is transcribed), separated by the non-coding control region (NCCR), a highly variable region (1) (Figure 2).

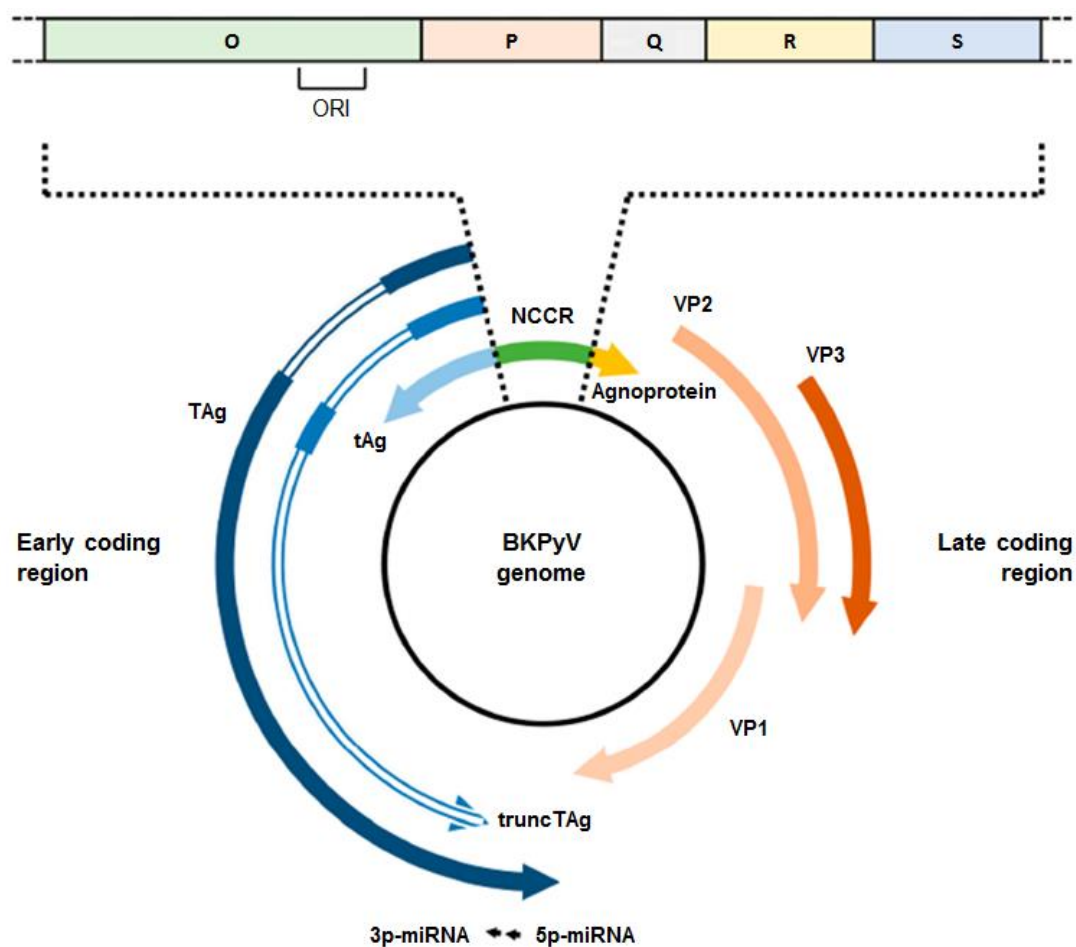


Figure 2. Structure of BKPyV genome. The BKPyV genome is a closed circular, double-stranded DNA (dsDNA) molecule comprising approximately 5 kbp. It is divided into two transcriptional regions, the early and the late coding regions, separated by the non-coding control region (NCCR). Transcription of both early and late coding regions proceeds in a bidirectional way from the origin of replication (ORI) within the NCCR. The early coding region encodes the large tumor antigen (TAg), the small tumor antigen (tAg) and the truncated TAg (truncTAg). The late coding region encodes the structural proteins (VP1, VP2, and VP3) as well as the agnoprotein. The late coding region also encodes for two microRNA (miRNA), 5p-miRNA and 3p-miRNA, produced after processing of a common precursor-miRNA (pre-miRNA). (Adapted from Helle, et al., 2017)

Within the NCCR is located the origin of replication (ORI), from where the replication proceeds in a bidirectional way, and also promoters and regulatory regions of early and late gene transcription. The early region encodes for three proteins, the large tumor antigen (TAg), the small tumor antigen (tAg) and the truncated TAg (truncTAg), that are expressed prior to DNA replication. The late region is only transcribed after the onset of DNA replication, and encodes for the structural proteins VP1, VP2 and VP3, as well as the agnoprotein (15,16). The late coding region also encodes for two microRNA (miRNA), referred to as 5p-miRNA and 3p-miRNA, produced after processing of a common precursor-miRNA (pre-miRNA) perfectly complementary to the 3' end of the TAg messenger RNA (mRNA) (17).

1.1.2.1. Genotypes and variants

BKPyV can be divided into four genotypes (I to IV) due to variations on the genomic region coding for VP1 (18). Genotype I is further divided into the subgroups Ia, Ib1, Ib2, and Ic (19), and genotype IV into subgroups IVa1, IVa2, IVb1, IVb2, IVc1, and IVc2 (20). The most prevalent is genotype I, distributed worldwide, followed by genotype IV which is found in East Asia and Europe, while genotypes II and III are less commonly detected (21). It has been demonstrated that genotypes II, III, and IV and subgroups Ib1 and Ib2 behave as five fully distinct serotypes. The implications of infection with the different genotypes are yet to be understood, but successive infections with different genotypes are possible as they don't cross-neutralize (18).

Besides from the variations on the VP1 region, variations on the NCCR lead to two different forms of BKPyV, referred to as archetype and rearranged variants. Archetype-NCCR is divided into five sequence blocks (Figure 2) named O, which contains the ORI, and P, Q, R and S, which contain regulatory regions for gene transcription (22). Rearrangements in the NCCR during viral replication usually involve duplications and deletions leading to the appearance of different rearranged-NCCR. These variants are more commonly found in the plasma and tissues of individuals with BKPyV-associated diseases (22,23). Archetypal variants of BKPyV are predominant in the urine, and are thought to be the transmissible forms of the virus, as they can be found in both healthy and diseased individuals (24). While the role of these variants in the development of BKPyV-associated diseases is not well established, it has already been shown that unlike rearranged strains, archetypal forms of BKPyV replicate poorly in cell culture (25). More recent studies have demonstrated that rearrangements on the NCCR lead to the decrease of viral miRNA production. This could result in an increase on the viral replication through the increase of TAg expression, since miRNAs are known to target TAg mRNAs to degradation (17). In this context, it has been

proposed that rearrangements in the NCCR might constitute an adaptation of the virus to enhance viral replication.

1.1.3. BKPyV replication cycle

The infection with BKPyV starts with the attachment of the virion to the host cell, followed by entry, genome expression and replication, assembly and release of the viral progeny (Figure 3).

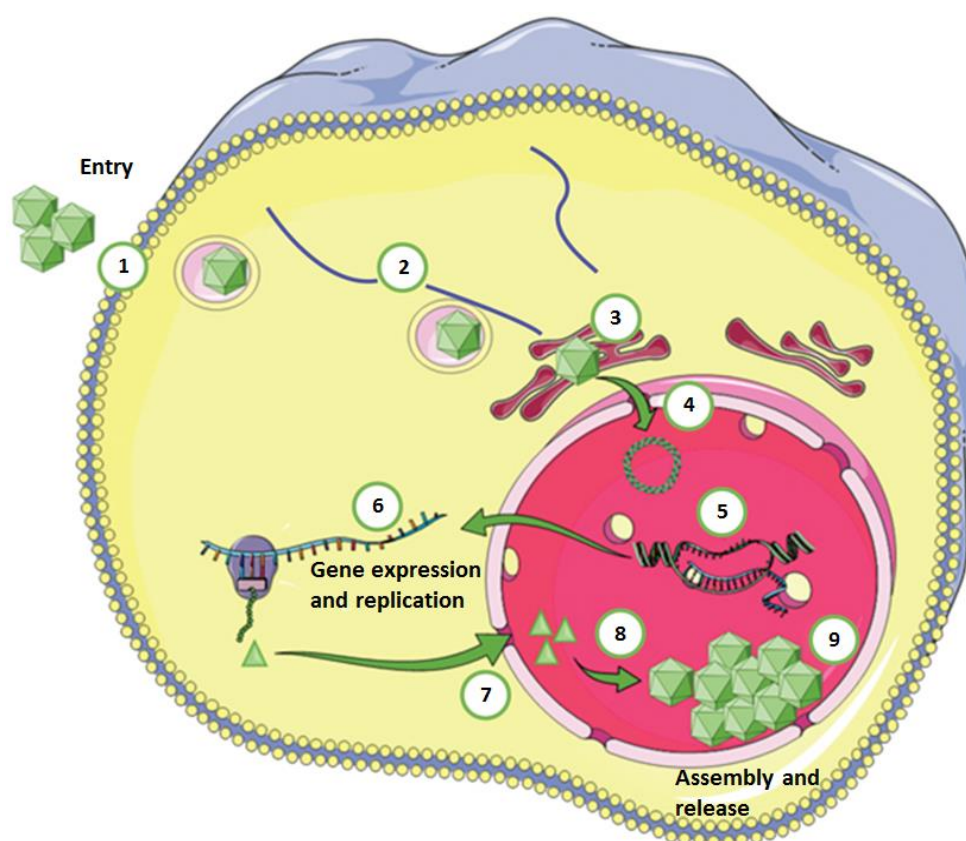


Figure 3. Schematic representation of BKPyV replication cycle. BKPyV infection starts with the attachment of the virions to the host cell by binding to the ganglioside receptors (mainly GT1b and GD1b). Then, the virions are internalized through a caveolae-mediated endocytic pathway (1). Inside the cell, the virus is transported along the microtubules towards the endoplasmic reticulum (ER; 2) where the uncoating process begins (3). The partially uncoated viruses subsequently gain access to the cytosol and the viral genome is transported into the nucleus through the nuclear pore complex due to VP2 and VP3 nuclear localization sequences (NLS) and the importin α/β 1-mediated import pathway (4). Once the viral genome enters the nucleus, the early gene expression begins. Early proteins are then translocated to the nucleus promoting genome replication and late gene expression (5). After translation of viral mRNAs in the cytosol (6), VP1, VP2 and VP3 gain access to the nucleus where they self-assemble around newly synthesized viral DNA (8). Progeny virions start to accumulate in the nucleus (9) and their release from infected cells is thought to occur mainly upon cell lysis. However, a non-lytic egress as also been described. (Adapted from Solis, et al., 2019)

Entry of the virus

In order to start the infection, the viral particle must attach to the host cell through the binding of viral VP1 with specific cell receptors. These receptors have been identified as gangliosides, one type of glycosphingolipids, composed of a ceramide chain and an oligosaccharide to which one or more sialic acid residues are linked. $\alpha(2,8)$ -disialic acid motif on b-series gangliosides has shown to be required for this interaction, thus BKPyV can interact with the gangliosides GT1b, GD1b, GD2 and GD3 (26,27). Despite this, it has already been demonstrated that BKPyV genotype IV is capable of establishing an infection through a ganglioside-independent pathway, showing that different BKPyV genotypes might present different cellular tropisms (18).

Following attachment to the cell surface, BKPyV is internalized through a caveolae-mediated endocytic pathway. Inside the cell, the virus is transported along the microtubules towards the endoplasmic reticulum (ER) where the uncoating process begins. In this process there seems to be the involvement of the ER protein disulfide isomerase which promotes conformational changes in VP1 molecules, providing the initial step for the capsid disassembly (28). Furthermore, proteins of the ER-associated degradation (ERAD) pathway, that are normally involved in the removal of misfolded proteins from the ER for proteasomal degradation, have been implicated as a mechanism used by the virus to escape from the ER to the cytosol (29).

Once in the cytosol and after partial uncoating, nuclear localization sequences (NLS) of VP2 and VP3 are exposed and thought to be used by the importin α/β 1-mediated import pathway to transport the viral genome into the nucleus through the nuclear pore complex. However, an alternative route for genome entry into the nucleus has also been suggested to be present, as the BKPyV NLS mutation only attenuates the infection (30).

Gene expression and genome replication

When in the nucleus, the viral genome remains episomal in host cells (11). Soon after the genome enters the nucleus, cellular transcription factors bind to the early promotor leading to the expression of early proteins. After translation in the cytosol, TAg is transported into the nucleus where it binds to the ORI, leading to the recruitment of the cellular replication complex and to the unwinding of the double stranded DNA through its helicase activity, mediating the initiation of BKPyV genome replication. Additionally, TAg stimulates cell cycle progression and prevents apoptosis, by binding to the tumor suppressor proteins pRb, p107 and p130, and p53, respectively (31). tAg has also been associated with cell cycle

progression through inactivation of the protein phosphatase 2A (32). After the onset of DNA replication, in a process promoted by TAg, late genes are transcribed leading to the expression of the structural proteins and also agnoprotein (16). Agnoprotein is a small regulatory protein which seems to play important roles during the viral replication cycle, including regulation of gene expression, genome replication, and also virion assembly and release (33).

Assembly and release of viral particles

After translation in the cytosol, the structural proteins VP1, VP2 and VP3 are imported into the nucleus where the assembly of new viral particles takes place. At 2 days post-infection (dpi), progeny virus start to accumulate in the nucleus, forming nuclear inclusions that can be seen by electron microscopy in tubular epithelial cells from kidney biopsies or excreted in the urine as decoy cells, which are virally infected epithelial cells characterized by large nuclear viral inclusions (34,35).

As for the release of the newly formed virus from infected cells, the knowledge is still very limited. Generally, it is suggested that the virus egress from the cell occurs by passive means such as host cell lysis, which has already been observed in RPTEC (human renal proximal tubular epithelial cells) (34). However BKPyV-infected cells rarely exhibit strong cytopathic effects (36). Furthermore, non-lytic egress for BKPyV progeny, although in smaller scale, has already been demonstrated (37). Even though further studies are needed in this subject, evidence points out that both lytical and non-lytical release of BKPyV progeny might occur.

1.2. Primary infection, latency and reactivation

BKPyV infection is ubiquitous, with a seroprevalence of over 80% among the adult population. Primary infection typically occurs during early childhood, with studies having shown that by the age of 10 approximately 70% of children present detectable serum antibodies against BKPyV (38). This primary infection is usually asymptomatic or associated with mild upper respiratory symptoms. The route of transmission is not yet fully established, but evidences of a primary upper respiratory infection, particularly in the tonsils, suggests that the virus is most likely transmitted through a respiratory route (39). However, other routes of transmission are also being proposed including fecal-oral, urino-oral, blood transfusion or organ transplantation (16,40). From the respiratory tract, presumably following a primary viremia, the virus is thought to disseminate to other sites of infection, including

mainly the urinary tract and the kidney. Following primary infection, BKPyV establishes a lifelong persistent infection mainly on epithelial cells of the urinary tract and the kidney, however, it is still unclear if the virus persists with a minimal replication, or if it enters a true viral latency. Occasional reactivations of the persistent virus occur that manifest as asymptomatic viruria with BKPyV loads of $<5 \log_{10}$ copies (c)/mL (41,42).

In immunocompetent individuals, the virus remains clinically silent throughout the life of the host. However, under immunosuppressing conditions, typically immunosuppressive disease or treatment, viral reactivation might occur. The major clinical complications of BKPyV reactivation are PyVAN and hemorrhagic cystitis in kidney and hematopoietic stem cell transplant recipients, respectively (5). Less commonly BKPyV-associated clinical manifestations include interstitial pneumonitis, retinitis, native kidney nephritis and meningoencephalitis, described particularly in severely immunocompromised individuals such as patients with acquired immune deficiency syndrome (43).

1.3. Polyomavirus-associated nephropathy

PyVAN most commonly occurs in kidney transplant recipients due to BKPyV reactivation, with a prevalence rate that ranges from 1 to 10% in these patients (44). After reactivation, BKPyV replication in tubular epithelial cells results in cytopathic changes in those cells. The progress of the infection leads to tubular cell injury and interstitial inflammation that characterize PyVAN. Persistent PyVAN eventually leads to tubular atrophy, interstitial fibrosis and loss of function(45), which can potentially result in graft loss in 10 to 80% of the cases (46).

Several factors have been described to modify the risk of BKPyV reactivation. However, immunosuppressive therapy is thought to be the one that has the major role in this reactivation. Rather than a specific therapy, it has been described that the overall degree of immunosuppression is responsible for increasing the risk. In fact, the development of more potent immunosuppressant drugs, such as tacrolimus and mycophenolate mofetil, have led to an increase on the PyVAN incidence rate in kidney transplant recipients (47).

Other than immunosuppressive therapy, a variety of weekly associated risk factors have also been described to increase BKPyV reactivation. The preferential occurrence of PyVAN after kidney transplants over other solid organ transplants, even with comparable levels of immunosuppression (48), points out that the process of kidney transplantation itself and the resultant renal injury, might have a role on BKPyV reactivation. This includes prolonged cold ischemia time, delayed graft function and ureteral stent placement. Other transplant-related

risk factors that are usually referred to include degree of human leukocyte antigen (HLA) mismatch, donor BKPyV seropositivity, recipient BKPyV seronegativity, donor and recipient older age, recipient male gender and previous graft loss due to PyVAN. Prolonged pre-transplant dialysis has also been pointed out as a potential risk factor for PyVAN development (5,16,49).

Interestingly, the donor's BKPyV strain reactivation has been suggested to be the one involved on PyVAN development. Consequently, genotype mismatch between the donor and the recipient neutralization profiles and their subsequently replicating strain before transplant might significantly increase the risk of BKPyV reactivation (50).

1.3.1. Early screening and diagnosis

Clinically BKPyV reactivation is asymptomatic, and in case of PyVAN development it only manifests as a deterioration in allograft function. The first evidence of BKPyV reactivation is a high-level BKPyV viruria with decoy cells shedding, followed by viremia (51). In kidney transplant recipients it is estimated that approximately 50% of the patients who present high-level viruria evolve to viremia 2 to 6 weeks later, and that 50% of those who develop viremia are diagnosed with PyVAN 2 to 6 weeks later (52).

Because no efficient and safe antiviral therapies against BKPyV are yet available, screening for BKPyV replication in urine and blood has become the key recommendation for kidney transplant recipients. This allows an early intervention, which to date consists mainly in modulating the immunosuppressive treatment, in order to prevent the development of PyVAN and ultimately graft loss (52).

According to the Guidelines from the Infectious Diseases Community of Practice of the American Society of Transplantation, current recommendations point that kidney transplant recipients should be screened for BKPyV DNA loads in plasma by quantitative polymerase chain reaction (PCR)-based techniques, first monthly until month 9, then every 3 months until 2 years post-transplant. This screening allows the identification of patients that should be considered for a preemptive treatment for PyVAN, however, prior to reducing immunosuppression and risking the precipitation of acute rejection, BKPyV viremia should be confirmed within 3 weeks. BKPyV DNA plasma loads sustained above 3 log₁₀ c/mL, but below 4 log₁₀ c/mL define probable PyVAN, and above 4 log₁₀ c/mL define presumptive PyVAN. Screening for BKPyV reactivation can also be performed in the urine, although these methods are usually less preferred because of their low positive predictive values. In this case, either urine cytology for decoy cells or BKPyV DNA loads in urine by quantitative PCR-

based techniques can be performed, 2 times a week for the first 3 months, then monthly until month 6, then every 3 months until 2 years post-transplant. If decoy cells are detected or if urine BKPyV DNA loads increase above 7 log₁₀ c/mL, patients should be tested for BKPyV viremia (52).

Although a BKPyV DNA plasma load above 4 log₁₀ c/mL can diagnose a presumptive PyVAN, kidney biopsy remains the gold standard for definitive diagnosis of PyVAN. Because of the focal nature of the infection, it is recommended that a minimum of two biopsy cores are taken, preferentially containing medullary tissue, which is a significant reservoir for BKPyV infection, in order to avoid false negatives. The diagnosis of PyVAN is made upon identification of cytopathic changes in the tubular epithelial cells of the biopsy tissue. Since PyVAN and acute rejection might resemble morphologically, the diagnosis can be confirmed by immunohistochemistry or *in situ* hybridization (52).

1.3.2. Therapeutic approaches

Because BKPyV lacks a specific target, the search for a specific and safe antiviral against this virus becomes more difficult, and to date, none is still available. As such, the mainstay approach for persistent BKPyV replication and PyVAN in kidney transplant recipients is to reduce the immunosuppressive treatment. This strategy allows the reconstitution of the patients' immune system, which becomes capable of controlling the infection. Although not all patients exhibit the same response, an efficient clearance of BKPyV viremia and the preservation of allograft function has been described in a high percentage of patients in several studies (52,53). Despite this, by reducing immunosuppression there is a risk of precipitating acute rejection. To prevent this from occurring, the immunosuppression reduction must be done in a stepwise manner with close monitoring of serum creatinine levels and BKPyV viremia, at least every two weeks until viremia clearance (52).

The immunosuppressive regimen for kidney transplant recipients may vary between centers but usually consists of a calcineurin inhibitor, such as cyclosporine A and tacrolimus, an antimetabolite, such as azathioprine and mycophenolate mofetil, and a corticosteroid (54). Regarding the adjustment of the immunosuppressive treatment, there is no single approach and different strategies have been suggested. One of these approaches consists to first reduce the calcineurin inhibitor or the antiproliferative drug, followed by a dose reduction of the other drug and then discontinuation of the antiproliferative drug (52). Alternatively, other approaches include switching to less potent drugs, like for example, from tacrolimus to cyclosporine A (a less potent calcineurin inhibitor) (55), from the calcineurin inhibitor to

sirolimus, or from mycophenolate mofetil to sirolimus (56,57) or leflunomide (58–60). Successful outcomes have been reported with all these different strategies, however, that is still no randomized controlled trial that can recommend one over the others.

In addition to the adjustments in the immunosuppressive treatment, and despite no specific antiviral against BKPyV is yet available, the off-label use of agents with potential anti-BKPyV activity in patients with PyVAN has been reported. These includes cidofovir, fluoroquinolones and intravenous immunoglobulins (IVIGs). However, as they are often used in combination with immunosuppression reduction and randomized controlled trials are still lacking, it is difficult to make strong conclusions about their efficacy (61).

Novel therapeutic approaches against BKPyV are starting to appear. These include adoptive T cell therapy, that is starting to be studied to treat BKPyV-associated diseases (62,63). Furthermore, a vaccine for prevention of cytomegalovirus and BKPyV reactivation is currently under evaluation on a phase I clinical trial (64).

Leflunomide

Leflunomide presents both immunosuppressive and antiviral properties, being currently approved for the treatment of rheumatoid arthritis. Its antiviral effects are attributed to its active metabolite A771726 which inhibits protein kinase activity and the synthesis of pyrimidines. *In vitro*, leflunomide has already shown to interfere with BKPyV replication in RPTEC (65). Several studies have reported the use of leflunomide, often combined with reduction of the immunosuppressive treatment, and clearance or reduction of BKPyV viremia has been described (58–60). However, determining its efficacy remains difficult since therapeutic outcomes change between studies (53) and no randomized controlled trials have been performed. Additionally, significant toxic effects have been described when using this drug including hepatitis, hemolysis, thrombotic microangiopathy, bone marrow suppression, and fungal pneumonia (52).

Mammalian target of rapamycin inhibitors

Mammalian target of rapamycin (mTOR) inhibitors, such as sirolimus and everolimus, are immunosuppressive agents that also exhibit antitumoral activity by inhibiting cell-cycle progression and proliferation. *In vitro*, they have shown to decrease BKPyV replication by interfering with mTOR–SP6-kinase activation (66). Different studies have reported that

kidney transplant recipients receiving mTOR inhibitors compared to those receiving standard immunosuppressive regimens exhibit a more efficient clearance of BKPyV viruria and viremia or even lower risks of developing BKPyV viremia (56,57,67). Although its use seems promising in preventing PyVAN, further studies are needed, particularly regarding their toxicity, as severe adverse effects have been reported, including pneumonitis and proteinuria (68,69).

Cidofovir

Cidofovir is a nucleoside analog and viral DNA polymerase inhibitor only approved for the treatment of cytomegalovirus retinitis. The antiviral effect against BKPyV has already been demonstrated *in vitro* in RPTEC, however, the mechanism behind this effect remains unclear (70). Treating kidney transplant recipients with cidofovir alongside with reducing immunosuppression has shown favorable outcomes in some studies (71,72), whereas others claim no advantage with this treatment (53). Despite this potential benefit in PyVAN, major adverse effects associated with the use of this drug include nephrotoxicity and uveitis, with the first one limiting its use in kidney transplant recipients (52,73).

Brincidofovir is an experimental pro-drug of cidofovir, orally administrated and substantially less nephrotoxic. *In vitro*, it has shown to decrease BKPyV replication in RPTEC more efficiently when compared to cidofovir, and also to exhibit fewer toxicity (74). Successful outcomes have been obtained with this drug in two different studies (75,76), however, randomized controlled trials are currently on hold due to adverse events already described (52).

Fluoroquinolones

Fluoroquinolones are a family of broad-spectrum antibiotics that have shown to present an antiviral effect against BKPyV *in vitro*, possibly by inhibiting the helicase activity of TAg (77). Initial studies have reported a prophylactic effect with the use of fluoroquinolones in both kidney and hematopoietic stem cell transplant recipients (78,79). However, a double-blind, placebo-controlled randomized trial failed to demonstrate the efficacy of a 3-month course of levofloxacin on lowering BKPyV viruria, as well as it shown an increased risk of bacterial resistance (80). These results do not support the use of fluoroquinolones on the prevention of BKPyV reactivation.

Intravenous immunoglobulins

Because of the high seroprevalence of BKPyV in the population, commercially available IVIG preparations have been demonstrated to contain high titers of potent BKPyV neutralizing antibodies (NAb). Its antiviral effect is attributed to direct neutralizing activity but also to indirect immunomodulatory effects (81). Some studies have described the use of IVIG in combination with reduced immunosuppression with beneficial outcomes (82,83). Additionally, a study suggesting that higher NAb titers against the replicating strain of BKPyV were associated with a lower risk of BKPyV reactivation, supports the potential benefit of administering NAb either as a preventive or a therapeutic strategy (50). However, no controlled studies have yet been reported.

Cellular immunotherapy

Immunosuppression is thought to be the main cause of BKPyV reactivation. In this context, the adoptive transfer of BKPyV-reactive T cells may help to restore the immune response of these patients, thus allowing it to control viral replication and reducing the complications associated with this reactivation. This approach has already shown to be effective to treat other virus-associated diseases, particularly associated with Epstein-Barr virus and cytomegalovirus (84,85), in immunocompromised patients. More recently, the use of this immunotherapy started to be studied to treat diseases caused by BKPyV infection. The immunodominant epitopes of BKPyV to be used on T cell expansion are not yet defined, nevertheless, it is already reported the *in vitro* expansion of BKPyV-reactive T cells using overlapping peptide mixes of the five BKPyV proteins (VP1, VP2, VP4, TAg and tAg) (62). One study described the use of adoptive T cell therapy with full resolution of the symptoms and viremia on a patient that who, following hematopoietic stem cell transplantation, developed hemorrhagic cystitis due to BKPyV reactivation (63). Another promising study in this area described the use of adoptive transfer of virus-reactive T cells targeting BKPyV and four other viruses (Epstein-Barr virus, adenovirus, cytomegalovirus, and Human Herpesvirus 6) known to cause frequent infections in immunocompromised patients, with a great clinical response (86). With further research in this area, adoptive T cell therapy can become a potential approach to treat BKPyV-associated diseases.

1.4. Model systems for the study of BKPyV infection

To better understand the virus' structure, the replication cycle, the pathogenicity, and also to identify compounds with antiviral activity, both *in vitro* and *in vivo* models are crucial. Much of the knowledge regarding BKPyV in the past has been inferred from the much more studied polyomavirus SV40 (11,13). However, in the last years, a big effort to study BKPyV has been made as it is increasingly recognized as responsible for an important infection affecting mostly kidney and hematopoietic stem cell transplant recipients using the new potent immunosuppressive protocols.

To date, BKPyV has been known to replicate only in human and monkey cells. Vero cells were the first cells used to isolate BKPyV and currently, this African green monkey kidney cell line is still widely used in research to replicate the virus (3). RPTEC, however, constitute a more appropriate model for the study of BKPyV, as they are one of the major target cells of the virus *in vivo* (34). The problem regarding these cells is that primary RPTEC divide slowly and quickly enter into replicative senescence. Because of that, human telomerase reverse transcriptase (hTERT) immortalized RPTEC (RPTEC/TERT1), which have shown to maintain their functional characteristics, also started to being used (87). Other kidney cell lines of both human and monkey origin, human embryonic kidney 293 (HEK293) cells and CV-1 cells, respectively, are also used (88,89). Interestingly, other cell types allow the replication of BKPyV, including human salivary gland cells (90) and human embryonic lung fibroblast cells, such as MRC-5 (91). The cells mentioned above successfully allow the propagation of rearranged forms of BKPyV. Archetype forms, however, due to low TAg expression replicate poorly in cell culture. The human endothelial cell line HUV-EC-C was the first one to allow the propagation of archetypal BKPyV (25). More recently, archetype forms of BKPyV have also shown to be able to replicate in cell lines transformed with the SV40 TAg including 293TT and COS-7 cells, cell lines derived from HEK293 and CV-1 cells, respectively (24).

Regarding the virus, recombinant viruses with both rearranged and archetype NCCR are commonly used. Rearranged strains include Gardner, Dunlop and TU strains, and as for the archetypal strains, WW and WWT strains are among the most common (92).

Alternatively, virus-like particles (VLPs) can be used. VLPs consist on capsid-like particles that spontaneously self-assemble upon overexpression of the major capsid protein VP1, but that devoid of the virus' genetic material. These models can be produced in a variety of different systems including *Escherichia coli*, yeast, insect cells and mammalian cells (93). Additionally, these systems can be co-transfected with vectors encoding a reporter gene, enabling the quantification of the particles that enter target cells. Although these models cannot be used to study BKPyV replication, as they do not contain the viral genome, they

have provided useful information on the viral structure and on the early steps of the viral cycle, including interactions with cell surface receptors and entry (13,94). Finally, VLPs seem to have a promising application in the development of vaccines that could be used prior to transplantation (93).

Although *in vitro* models are sufficient to provide information on the virus replication cycle, animal models are needed in order to better understand the mechanisms underlying the virus-associated diseases but also for conducting preclinical tests on potential new antiviral drugs and vaccines. Because of the narrow host range of BKPyV the development of small animal models to study BKPyV infection was hampered. In fact, BKPyV DNA replication is inhibited by a restriction factor found in murine cells (95). Previous attempts to develop an animal model for BKPyV infection have already been carried out (91). However, an animal model that closely mimics BKPyV infection in humans is still lacking.

2. Aims of this study

Over the last years, the development of more potent immunosuppressive agents has made BKPyV emerge as an important pathogen responsible for serious complications such as PyVAN in kidney transplant recipients. The lack of specific and safe antiviral agents that can efficiently stop BKPyV replication still poses significant challenges, as PyVAN can lead to alterations in the kidney's structure, loss of function and potentially graft loss.

In this context, this project focused on studying the efficacy of two drugs - NA-17 and TN-18 - against BKPyV. For that purpose, this study was divided in different tasks in order to provide a characterization of the drugs' effect against BKPyV.

First, the effect of both drugs was assessed on Vero cells, a monkey kidney cell line, to determine whether they had potential to proceed for further testing. Then, the drugs that exhibited an antiviral effect on the first step, were submitted to a more detailed study on a better *in vitro* model of the virus target *in vivo*, RPTEC/TERT1. On this second stage a selected range of concentrations of each drug was used to characterize their effect against the virus. The inhibition of BKPyV infection for each concentration of both drugs was determined and used to calculate the respective effective concentrations 50% (EC₅₀). Then, as both drugs were known to exhibit an antimetabolite effect, the alterations on the extracellular BKPyV DNA load were measured for each concentration tested, and a correspondence was tried with the variations on the release of infectious progeny.

3. Materials and Methods

3.1. Cell culture

Vero cells (ATCC, CCL-81) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS). RPTEC/TERT1 (Evercyte, CHT-003-0002) were maintained in renal epithelial growth medium (REGM) supplemented with 0.1% recombinant human epidermal growth factor, 0.1% hydrocortisone, 0.1% epinephrine, 0.1% insulin, 0.1% triiodothyronine, 0.1% transferrin, 0.1% GA-100 (gentamicin and amphotericin B) and 0.5% FBS (REGM Bulletkit; Lonza). All cells were kept in a humidified incubator at 37°C with 5% CO₂ and propagated as described by the manufacturer.

3.2. Virus

3.2.1. Virus stock production

BKPyV stock was produced by transfecting Vero cells with BKPyV Dunlop genome. Briefly, the pUC19pBKv(34-2) plasmid, provided by Professor Walter Atwood (Brown University, USA), was amplified after transformation of chemically competent *Escherichia coli* (Invitrogen, C404010). The plasmids produced were digested with BamHI to separate BKPyV genome from the vector plasmid and were used to transfect Vero cells. Cells were kept in culture until cytopathic effects were observed. After that time, cell culture supernatants were harvested and submitted to 3 freeze/thaw cycles that allowed cell lysis and release of the virions. BKPyV was further amplified by successive infection of Vero cells, and the cell culture supernatant of infected cells was kept. The viral stock produced was stored at -80°C.

3.2.1. Virus stock titration

BKPyV stock was titrated in Vero cells and RPTEC/TERT1. For the titration, cells were plated and infected 24 h later with the viruses in 10-fold serial dilutions ranging from 10⁻¹ to 10⁻⁸. After 2 h of incubation for viral adsorption, the supernatants were removed and replaced with the adequate cell growth medium. At 72 h post-infection (hpi), cells were washed, fixed and immunostained as described below. The stocks' titer was expressed in 50% tissue culture infectious dose (TCID₅₀)/mL and determined using Reed and Muench calculator (96) (Brett D. Lindenbach, 2008).

3.3. Infection and drug treatment

Cells were plated in 96-well plates for confluence at day 5 and infected 24 h later with BKPyV Dunlop at a multiplicity of infection (MOI) of 1 in DMEM. After 2 h of incubation in a humidified incubator at 37°C with 5% CO₂, the supernatants were removed and replaced with the adequate cell growth medium (for Vero cells, medium supplementation with FBS was of 5%) with or without the drugs for 3 days. Controls containing only cells or only cells with the drugs were also included. All experiments were performed in triplicates.

Prior to each experiment, NA-17 and TN-18 drugs were freshly dissolved in dimethyl sulfoxide (DMSO) and H₂O, respectively. NA-17 was further diluted in cell growth medium with DMSO at 1:1000 in 10-fold serial dilutions ranging from 10 nM to 100 µM. TN-18 was further diluted in cell growth medium in 10-fold serial dilutions ranging from 10 nM to 1 mM. DMSO controls matching the DMSO concentration in 100 µM NA-17 (1:1000) were included in all experiments. The drugs' structures are protected under laboratory confidentiality and therefore, they are only represented by a code.

3.4. Immunofluorescence staining, microscopy, and digital image processing

Cells were washed with phosphate-buffered saline (PBS) at 72 hpi, fixed with 3.7% paraformaldehyde (PFA) in PBS for 10 min and permeabilized with 0.5% Triton X-100 in filtered CSK (cytoskeletal buffer; 10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EDTA) and H₂O (1:1) for 15 min, both at room temperature. Fixed cells were then incubated with the primary antibody for 1 h at room temperature, and the secondary antibody for 30 min at room temperature in the dark. The primary antibody used was mouse monoclonal anti-SV40 TAg (Pab416; 1:500; Abcam), and the secondary antibody used was goat anti-mouse IgG conjugated with Alexa Fluor Plus 488 (1:1000; Thermo Fisher Scientific), both diluted in PBS/Tween 0.1%. Cell nuclei were stained with DAPI (1:1000; Santa Cruz Biotechnology).

Images were collected using an inverted microscope (Axio Vert.A1 and Colibri 7 light source, Zeiss). For cell counting, pictures from two random fields per well were taken and DAPI and TAg stained nuclei were automatically counted using QuantIF (Figure 4), an ImageJ (National Institute of Health, Bethesda, MD, USA) macro (97).

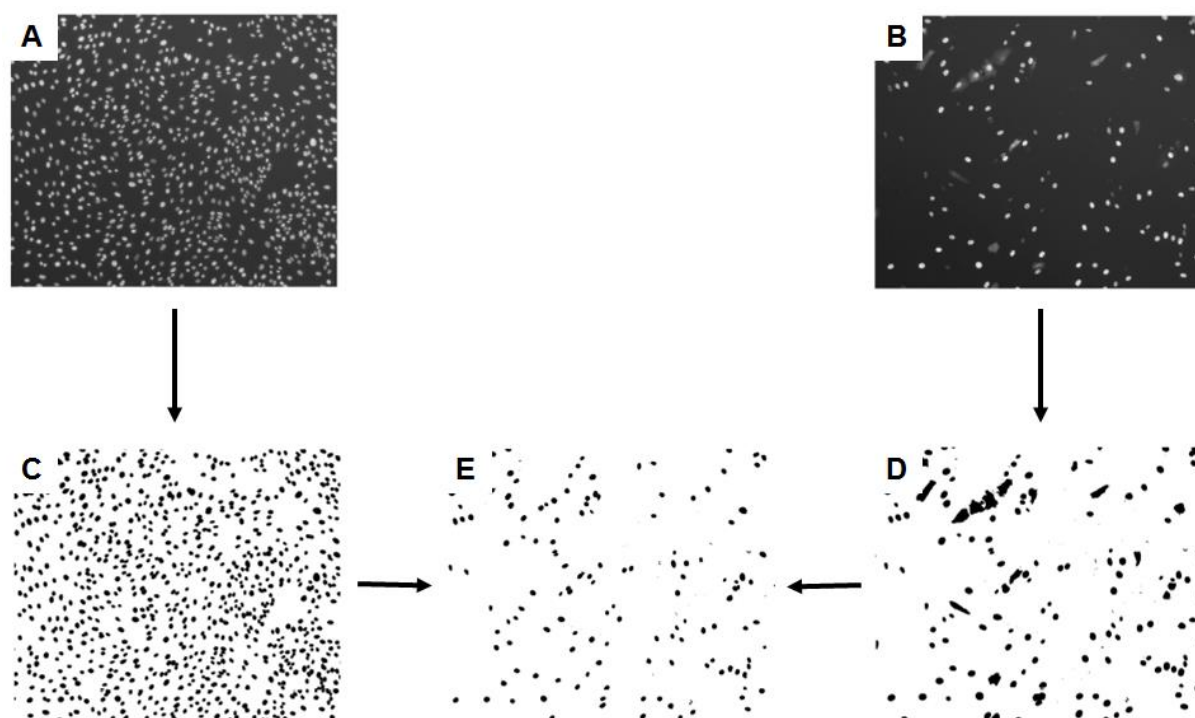


Figure 4. Description of the QuantIF macro. Two pictures of each field must be taken, one corresponding to the specific staining (infected cells) and the other corresponding to the DAPI staining (total cells), for analysis by the QuantIF macro. Both the DAPI staining image (A) and the specific staining image (B) are then converted to a staining mask, (C) and (D), respectively. A third mask corresponding to the nuclei of the immunostained cells is created (E). Finally, DAPI stained and immunostained cells' nuclei are counted using the DAPI and the immunostained cells' nuclei staining masks. (Adapted from Handala, et al., 2019)

3.5. Extracellular BKPyV DNA load

To quantify the extracellular BKPyV DNA loads, cell culture supernatants of infected cells both untreated and treated with the drugs were harvested at 72 hpi and stored at 4°C until quantification by quantitative real-time PCR (qPCR) using primers and a probe targeting a common region of the VP1 and VP2 genes in the BKPyV genome. Reactions were performed in a total volume of 25 µL containing 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 10 µM of each primer and the 6-carboxyfluorescein (FAM)-labeled probe, and 5 µL of supernatant. Amplification was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) with the following PCR program: initial step of 2 min at 50°C followed by 1 cycle at 95°C for 10 min, and 45 two-step cycles at 95°C for 15 s and 60°C for 1 min. BKPyV DNA loads were determined using a standard curve generated by amplification of four quantification standards containing different known concentrations of BKPyV DNA (9 000 000 to 9000 c/mL of the pUC19pBKv(34-2) plasmid in

10-fold serial dilutions). For every experimental condition triplicates were analyzed. A BKPyV-negative control was added to each run.

3.6. Release of infectious progeny

To detect the release of infectious progeny into the supernatant, cell culture supernatants of infected cells both treated and untreated with each drug were harvested at 72 hpi and stored at 4°C. Supernatants were used to infect new cells for 2 h and were then removed and replaced with fresh cell growth medium. At 72 hpi, cells were washed, fixed and immunostained as described above.

3.7. Calculation of the effective concentrations 50%

GraphPad Prism 8 software (GraphPad, La Jolla, CA) was used for curve fitting to determine the EC₅₀. EC₅₀ corresponds to the drug concentration responsible for 50% inhibition of BKPyV infection.

4. Results

4.1. Effect of NA-17 and TN-18 on BKPyV infection on Vero cells

4.1.1. Inhibition of BKPyV infection

First, the effect of both drugs, NA-17 and TN-18, on inhibiting BKPyV infection was evaluated on Vero cells. For such, Vero cells were infected with BKPyV and different concentrations of each drug were added 2 hpi. Cells were fixed at 72 hpi and immunostained for TAg. Cell nuclei were stained with DAPI. Then, pictures were taken for cell counting, and DAPI and TAg stained nuclei were automatically counted using QuantIF, an ImageJ macro. Results show an inhibition of BKPyV infection only with the highest concentration tested for each drug (Figure 5A and B). Of note, NA-17 at concentrations from 10 μ M and TN-18 at a concentration of 1000 μ M reduced the total cell number per field (Figure 5C and D).

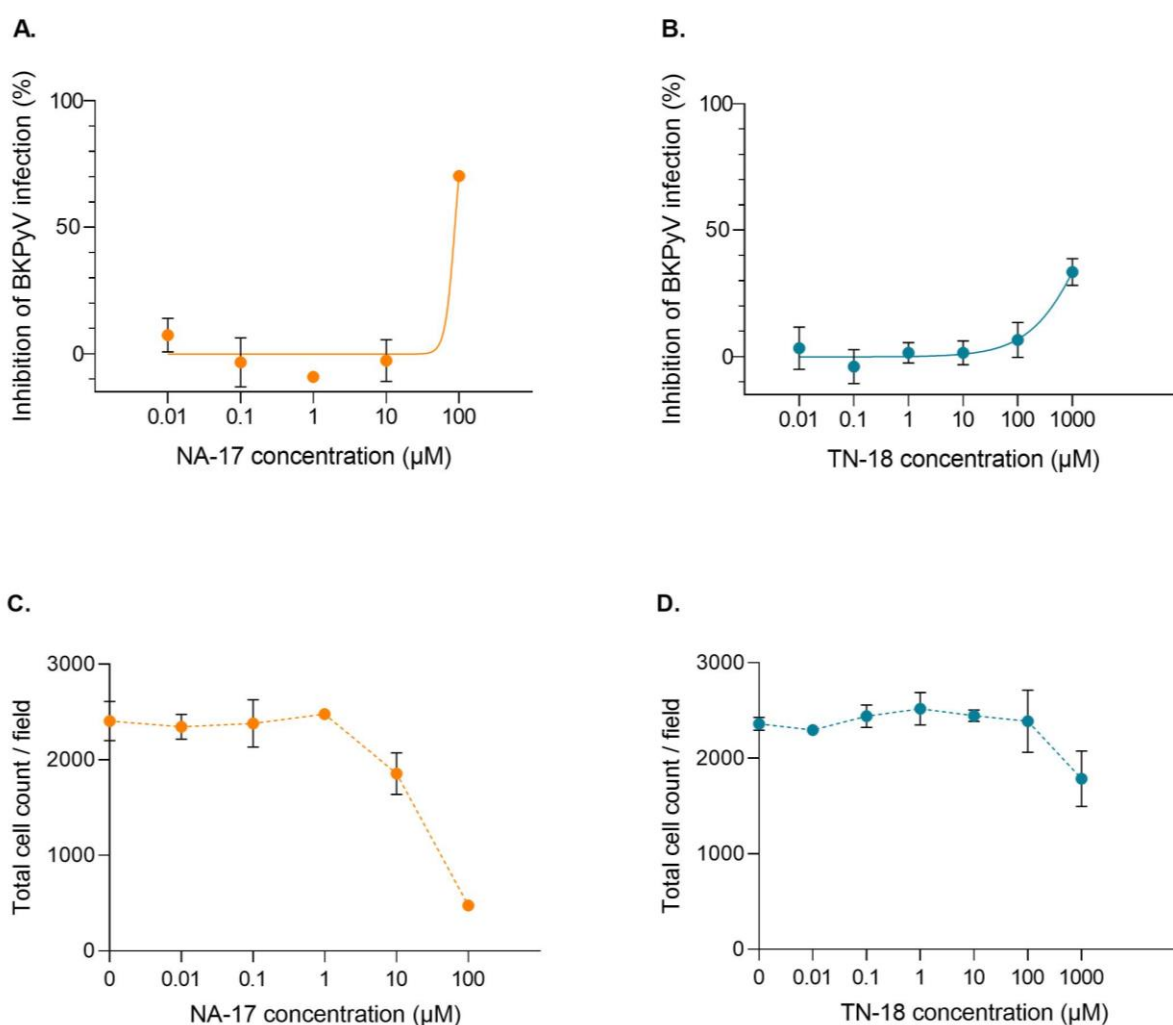


Figure 5. Effect of increasing concentrations of NA-17 and TN-18 on the inhibition of BKPyV infection on Vero cells. (A, B) Vero cells infected with BKPyV were treated 2 hpi with the indicated concentrations of NA-17 (A) and TN-18 (B). Cells were fixed at 72 hpi and immunostained for TAg. Cell nuclei were stained with DAPI. Pictures were taken for cell counting, and DAPI and TAg stained nuclei were automatically counted using QuantIF, an ImageJ macro. The percentage of inhibition of BKPyV infection for each concentration of the drugs was calculated in relation to a control of infected Vero cells without any treatment. Determinations were performed in triplicate, each corresponding to the analysis of two pictures. The mean values are shown, and the error bars represent the standard deviations of means between triplicates. Curve fitting was obtained using GraphPad Prism 8 software. (C, D) Total cell number of infected Vero cells both untreated and treated with different concentrations of NA-17 (C) and TN-18 (D). The number of cells in each picture was counted using QuantIF. The mean values are shown, and the error bars represent the standard deviations of means between triplicates.

Since an inhibition of BKPyV infection on Vero cells was observed for NA-17 and TN-18, the effect of both drugs was then investigated in RPTEC/TERT1, an immortalized cell line of the major target cells of BKPyV *in vivo*.

4.2. Effect of NA-17 and TN-18 on BKPyV infection on RPTEC/TERT1

4.2.1. Inhibition of BKPyV infection

To investigate the effect of both drugs on inhibiting BKPyV infection on RPTEC/TERT1, we proceeded like previously for Vero cells. Similarly to what we observed for Vero cells, the lowest concentrations tested for both drugs provided none to a very discrete effect. For NA-17 an inhibitory effect on BKPyV infection starts to be observed with a concentration of 10 μ M and seems to increase in a concentration dependent manner, as it increases with a concentration of 100 μ M. For TN-18, like previously observed on Vero cells, an antiviral effect is only evident for the highest concentration tested (Figure 6A and B). Of note, unlike observed on Vero cells, none of the concentrations tested for both drugs seems to have a significant impact on the total cell count (Figure 6C and D).

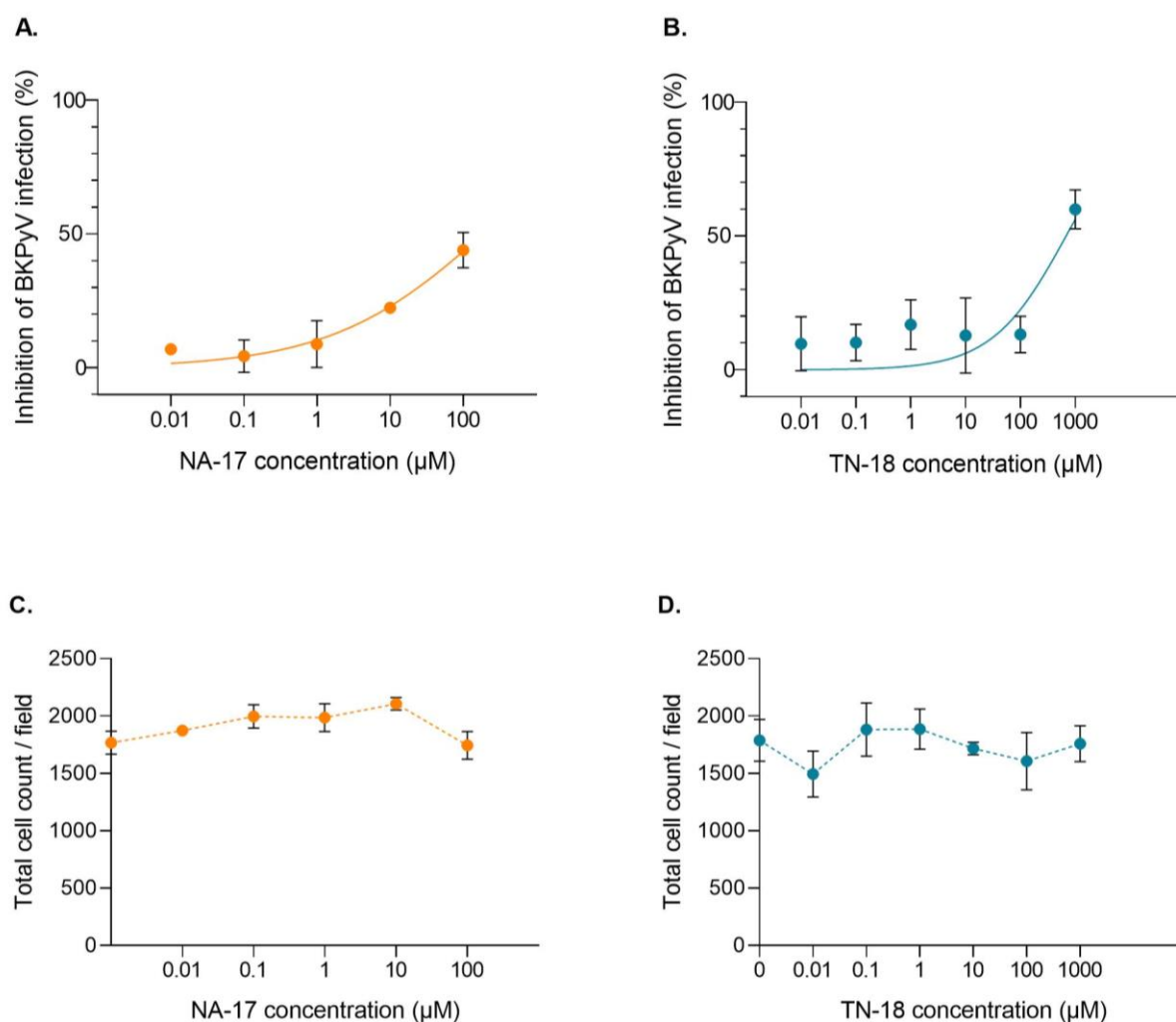


Figure 6. Effect of increasing concentrations of NA-17 and TN-18 on the inhibition of BKPyV infection on RPTEC/TERT1. (A, B) RPTEC/TERT1 infected with BKPyV were treated 2 hpi with the indicated concentrations of NA-17 (A) and TN-18 (B). Cells were fixed at 72 hpi and immunostained for TAg. Cell nuclei were stained with DAPI. Pictures were taken for cell counting, and DAPI and TAg stained nuclei were automatically counted using QuantIF, an ImageJ macro. The percentage of inhibition of BKPyV infection for each concentration of the drugs was calculated in relation to a control of infected RPTEC/TERT1 without any treatment. Determinations were performed in triplicate, each corresponding to the analysis of two pictures. The mean values are shown, and the error bars represent the standard deviations of means between triplicates. Curve fitting was obtained using GraphPad Prism 8 software. (C, D) Total cell number for infected RPTEC/TERT1 both untreated and treated with different concentrations of NA-17 (C) and TN-18 (D). The number of cells in each picture was counted using QuantIF. The mean values are shown, and the error bars represent the standard deviations of means between triplicates.

In order to determine the EC_{50} for both drugs, curve fitting based on the data obtained with the RPTEC/TERT1 was performed using GraphPad Prism 8 software (Figure 6). An EC_{50} of 190 μM and an EC_{50} of 682 μM were calculated for NA-17 and TN-18, respectively (Table 1).

Table 1. Calculated EC₅₀ values for NA-17 and TN-18 tested on RPTEC/TERT1^a

Drug	EC ₅₀ (μM) (95% CI)
NA-17	190 (97.9 - 566)
TN-18	682 (311 - 23 310)

^a EC₅₀ values and the correspondent 95% confidence intervals (CI) were calculated based on the curve fitting performed using GraphPad Prism 8 software

4.2.2. Effect on BKPyV genome replication

Although the mechanism by which these drugs may present an anti-BKPyV effect is not established, they are known to exhibit an antimetabolite effect. In this context, next, we investigated their effect on the virus genome replication by measuring the extracellular BKPyV DNA load. Cell culture supernatants of infected RPTEC/TERT1 both untreated and treated with NA-17 and TN-18 were harvested at 72 hpi and BKPyV DNA load was measured by qPCR. Supporting the results obtained with immunofluorescence, the lowest concentrations tested for both drugs don't seem to have any effect on inhibiting BKPyV replication (Figure 7). For NA-17 a decrease on BKPyV DNA load can be observed with the two highest concentrations. For TN-18 only the highest concentration seems to cause a decrease on BKPyV DNA load. Although an effect on viral DNA replication can be observed using the highest concentrations tested of these drugs, the decrease of extracellular BKPyV DNA load is very discrete, being for both cases <1 log₁₀ c/mL, reaching a maximum inhibition of approximately 5% and 7% for NA-17 and TN-18, respectively.

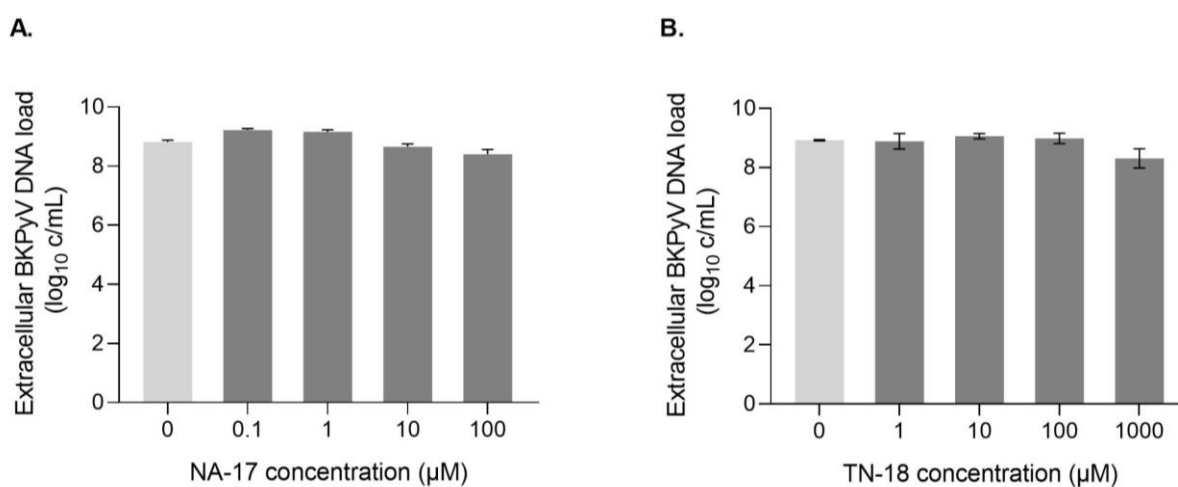


Figure 7. Effect of increasing concentrations of NA-17 and TN-18 on BKPyV DNA load. RPTEC/TERT1 infected with BKPyV were treated 2 hpi with the indicated concentrations of NA-17 (A) and TN-18 (B).

Supernatants were harvested at 72 hpi and BKPyV DNA load was measured by qPCR. Determinations were performed in triplicate. The mean values are shown, and the error bars represent the standard deviations.

4.2.3. Effect on the release of infectious progeny

To determine whether the decrease in extracellular BKPyV DNA load corresponded to the release of infectious progeny, supernatants harvested at 72 hpi from BKPyV-infected RPTEC/TERT1 untreated and treated with different concentrations of each drug were used to infect freshly seeded RPTEC/TERT1. Immunofluorescence staining was performed at 72 hpi like previously described and pictures were taken for cell counting. Results show an inhibition of infectious progeny release corresponding to the results found on extracellular BKPyV DNA load (Figure 8). This means that for NA-17 the release of infectious progeny decreased with the two highest concentrations tested, and for TN-18 only with the highest concentration. Of note, with the lowest concentrations tested of NA-17 and TN-18 an inhibition of approximately 20% and 40% in relation to the controls is already found, which does not correspond to the results obtained for the BKPyV DNA load, where differences from the controls were only observed with the highest concentrations. However, caution must be taken when analyzing these results since the infection of RPTEC/TERT1 using supernatants from both untreated and treated cells produced infection rates <1% for every case.

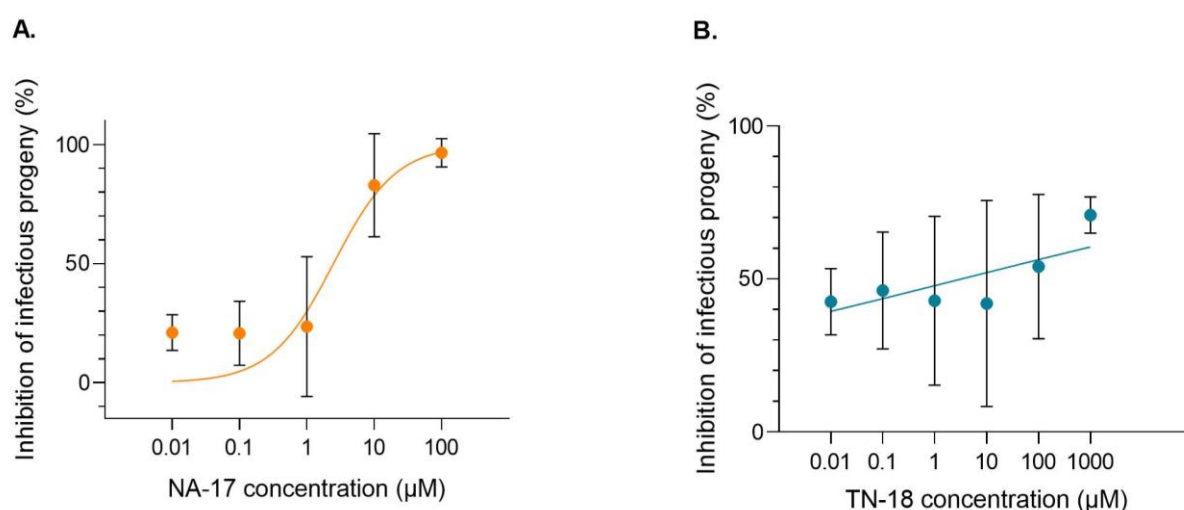


Figure 8. Effect of increasing concentrations of NA-17 and TN-18 on the release of infectious progeny. Supernatants harvested at 72 hpi from BKPyV-infected RPTEC/TERT1 untreated and treated with different concentrations of NA-17 (A) and TN-18 (B) were used to infect freshly seeded RPTEC/TERT1. Immunofluorescence staining for TAg was performed at 72 hpi. Cell nuclei were stained with DAPI. Pictures were taken for cell counting, and DAPI and TAg stained nuclei were automatically counted using QuantIF, an ImageJ macro. The percentage of inhibition of infectious progeny release for each concentration of the drugs was

calculated in relation to a control of fresh RPETC/TERT1 infected with the supernatants from untreated BKPyV-infected RPTEC/TERT1. Determinations were performed in triplicate, each corresponding to the analysis of two pictures. The mean values are shown, and the error bars represent the standard deviations of means between triplicates. Curve fitting was obtained using GraphPad Prism 8 software.

5. Discussion

A specific and safe antiviral that can efficiently stop BKPyV infection and prevent PyVAN progression is still in need. In this project, the effect of two drugs - NA-17 and TN-18 - against BKPyV replication was characterized in RPTEC/TERT1. We found out that the treatment with each drug 2 hpi resulted in a moderate inhibition of BKPyV infection, having been estimated EC_{50} values of 190 μ M and 682 μ M for NA-17 and TN-18, respectively. A more detailed study of the effect of these drugs against BKPyV showed that with the highest concentrations tested, both NA-17 and TN-18 were responsible for a decrease on the extracellular BKPyV DNA load, that although discrete, corresponded also with an inhibition on the release of infectious progeny.

Before proceeding to the study of the drugs on BKPyV-infected RPTEC/TERT1, a first assay was performed on Vero cells in order to determine if the drugs exhibited a potential antiviral effect against the virus. Vero cells were the first cells used to isolate BKPyV, and besides having shown to be permissive and to well replicate this virus, this cell line is easily cultivated, achieves confluence readily and does not require the use of specialized media (3,98). Because of this, this cell line is still widely used on basic research and constitutes an appropriate model for a first approach on evaluating the effect of a potential anti-BKPyV drug. First results obtained using Vero cells showed that 72 hpi an inhibition of BKPyV infection was obtained with 100 μ M of NA-17 and with 1000 μ M of TN-18. Although only the highest concentrations tested for each drug allowed an inhibition of the infection, a peak of BKPyV infection on Vero cells is expected between the 7 and 10 dpi (98), meaning that assays carried only until 3 dpi may not entirely reflect the effect of the drugs tested against the virus. As such, the anti-BKPyV effect observed in these preliminary results was considered sufficient to proceed with both drugs for further testing.

On a second stage, we wanted to characterize the anti-BKPyV effect of both drugs on a better *in vitro* model. As mentioned before, the ideal cells for this study would be RPTEC, one of the major targets of the virus *in vivo*, however, working with primary cells comes with its challenges as they are harder to cultivate and exhibit a limited lifespan. To overcome these problems, we decided to use RPTEC/TERT1, an immortalized cell line of RPTEC. To start the characterization of the drugs' effect on RPTEC/TERT1, the same assay previously performed on Vero cells was repeated on this cell line. Results obtained using RPTEC/TERT1 showed a better anti-BKPyV effect of both drugs on these cells. For NA-17 an inhibition of the infection was observed from a concentration of 10 μ M, that increased with the increase of the concentration. For TN-18, similarly to what we had observed on Vero cells, only the concentration of 1000 μ M seemed to have an impact on inhibiting the infection,

but a higher inhibition was obtained using this second cell type. Curve fitting to these results allowed to estimate the EC_{50} values for NA-17 and TN-18 of 190 μ M and 682 μ M, respectively. These EC_{50} values are relatively high, showing only a moderate antiviral effect, however, the CI shown for these values reflect the high variability of these results, making them hard to interpret. The range of concentrations tested for each drug did not allow to calculate the EC_{90} . Increasing these concentration ranges would provide more information on the drugs' efficacy.

As referred to before, although the mechanism by which these drugs may inhibit BKPyV infection is not established, both drugs are known to exhibit an antimetabolite effect. As such, it was expected that they interfered with viral genome replication. The inhibition of infection previously described for both drugs was determined with an immunofluorescence assay targeting TAg, which allowed to calculate the percentage of infection in each condition. However, TAg is a protein encoded by the early coding region of BKPyV genome, that is expressed prior to genome replication. For that reason, next, we decided to evaluate the effect of both drugs on viral genome replication by quantifying the extracellular BKPyV DNA load. We chose to use extracellular BKPyV DNA only, because BKPyV was already proven to be efficiently released from RPTEC, so a similar behavior was expected with RPETC/TERT1 (99). Moreover, extracellular DNA load had already been used in previous studies to evaluate the effect of antiviral drugs on BKPyV-infected RPTEC (74). These results supported the previous ones, as they showed a decrease in BKPyV DNA load for the two highest concentrations of NA-17 and for the highest concentration of TN-18. However, the decreases observed are very discrete, being for both cases $<1 \log_{10}$ c/mL when comparing to the control. To better understand these results it would have been interesting to quantify the input of BKPyV DNA load (100). This would tell us how much of the DNA measured was effectively released from infected cells as a result of viral replication.

In agreement with the decreases observed on BKPyV DNA load as a result of the treatment, an inhibition of infectious progeny release was found for the same concentrations. The main problem regarding these results is that low infection rates were obtained when using supernatants harvested 72 hpi to infect freshly seeded cells. The low number of infected cells increases the variability of the results obtained, making them hard to interpret. Furthermore, low infection rates were also obtained with the controls, most probably meaning that at 72 hpi the first replication cycle of the virus is not complete, and an efficient release of the progeny is not yet achieved. As described previously, the kinetics of BKPyV growth in RPTEC shows an increase on the extracellular DNA load at 3 dpi, reaching its peak at around 4 dpi (34,70). Because of this, a time point of 72 hpi has been largely used in studies regarding the evaluation of the antiviral effect of different drugs against BKPyV (70,74,77,100), which was

also chosen for our study. The results obtained, however, suggest a different kinetics of BKPyV infection on RPTEC/TERT1. As such, a study of the replication cycle of BKPyV on this cell line would help to better design the antiviral assay.

In what concerns the cytotoxic effects of NA-17 and TN-18 a detailed study was not conducted. However, to provide a first impression on the potential toxicity of these drugs, the cell count obtained under each condition was included in the results. The cell counts on RPTEC/TERT1 showed that with the range of concentrations tested for each drug, a significant effect on cell number was not observed. Nevertheless, in order to take strong conclusions on their toxicity, more specific assays must be conducted. As the exact mechanism by which the drugs interfere with the infection is not known, different cytotoxicity assays should be performed. Taking in consideration the known antimetabolite effect of both drugs and knowing that BKPyV genome replication is dependent on cellular DNA polymerases, these should include the effect of both drugs on cellular DNA replication, for example by measuring bromodeoxyuridine incorporation. This will allow to calculate the cytotoxic concentrations 50% and 90% (CC_{50} and CC_{90}), that together with the EC_{50} and the EC_{90} , can be used to calculate the corresponding selectivity indexes (SI, calculated by dividing the respective CC by the EC). The SI is a much better parameter to evaluate the drugs' efficacy *in vitro*, providing information on the selectivity of their antiviral effect.

In addition to all the assays performed, to complete the characterization of the antiviral effect of these drugs it would be interesting to evaluate early and late gene expression. It would be particularly interesting to evaluate the impact on late proteins, such as VP1 or agnoprotein, as they are expressed only after viral genome replication takes place. This could be achieved for example by measuring mRNA levels by reverse transcription qPCR (RT-qPCR) or by performing Western blot, preferentially in different time points to provide an insight on the drugs' temporal effect.

Regarding the cell type used, we chose to use RPTEC/TERT1. Using this cell line gave us an advantage as it maintains the characteristics of primary RPTEC, the major target cells of the virus *in vivo*, while overcoming the limited lifespan of primary cells in culture. However, the lentivirus vector used to express the hTERT in this cell line contains SV40 genome sequences, including the ORI. These SV40 sequences could interfere with our assays due to their ability to replicate in the presence of BKPyV (101). In this context, the results obtained with this cell line might not entirely reflect the effect of our drugs on BKPyV-infected RPTEC.

Overall our results show a moderate effect of both drugs tested against BKPyV infection. Results from the mathematical modeling of BKPyV replication in kidney transplant recipients show that an inhibition of over 80% of BKPyV replication must be achieved in order to obtain

a clearance of plasma and urine viral loads (102). The range of concentrations used for each drug allowed a maximum inhibition of infection of around 50%. Increasing these concentrations may allow to reach a higher inhibition of BKPyV infection. However, with higher concentrations more cytotoxic effects might appear, and the administration of such high concentrations *in vivo* may not be feasible. All together these results make us believe that the use of these drugs to stop BKPyV infection is limited, as other drugs have already shown a higher efficacy against BKPyV *in vitro* (74,77,100,103).

6. Conclusions and Future Outlooks

BKPyV is considered ubiquitous among the population, and despite it establishes a lifelong persistent infection in the host, in immunocompetent individuals, it remains clinically silent throughout their lives. Under immunosuppressing conditions, however, viral reactivation might occur, being PyVAN one of the major clinical complications of BKPyV reactivation in kidney transplant recipients. In order to prevent the progression of the infection and the eventual graft loss, and since an efficient and safe antiviral against BKPyV is still lacking, the mainstay approach is to decrease the immunosuppressive treatment, allowing the patient's immune system to fight the infection. Nevertheless, this approach still comes with its risks as it does not guarantee an efficient clearance of BKPyV infection and can also precipitate an acute graft rejection. In addition to these adjustments in the immunosuppressive treatment, the off-label use of some agents with potential anti-BKPyV activity has been reported. And although some promising outcomes have already been described, the lack of randomized controlled trials makes it difficult to take strong conclusions about their efficacy. In this context the development and search for antiviral drugs that can efficiently stop BKPyV replication and improve clinical outcomes in PyVAN after kidney transplantation, has become of great interest.

Taken together, the results from this study showed a moderate effect for both NA-17 and TN-18 against BKPyV, with NA-17 exhibiting a relatively stronger effect. The treatment of BKPyV-infected RPETC/TERT1 with these drugs allowed, with the highest concentrations tested, an inhibition of BKPyV infection, and a decrease on viral DNA load and infectious progeny release.

To evaluate these drugs' activity against BKPyV we decided to evaluate their effect on viral DNA load, as an antimetabolite effect was expected with both drugs. However, to obtain a more detailed characterization of their antiviral activity and to better understand the mechanism by which they interfere with BKPyV replication, their effect on early and late gene expression could also be evaluated.

As referred to before, other drugs have already been tested *in vitro* against BKPyV with promising results. In this study, the EC₅₀ values estimated were of 190 μ M and 682 μ M for NA-17 and TN-18, respectively, which are considerably high, making us believe that these drugs would have a limited effect *in vivo*. Nevertheless, it would be important to calculate the SI in order to understand the selectivity of the drugs' antiviral effect. To do so it would be needed to increase the concentrations range, that would allow us to also calculate the EC₉₀, and to include cytotoxicity assays, that would allow to calculate the CC₅₀ and CC₉₀ values.

As discussed before, different cells can be used to replicate BKPyV. Nevertheless, different cells replicate differently and replicate BKPyV differently. As such, the cell number to be plated and the time of infection needed can be quite different when working with different cells. Knowing the virus replication cycle on the cell type to be used is of extreme importance prior to conducting an assay to evaluate a drug's efficacy. One of the best *in vitro* models to study BKPyV infection is RPTEC. However, working with primary cells comes with its challenges, and many times cell lines are used instead. In this study we chose to work with RPTEC/TERT1, which as discussed before could have interfered with our results as the lentivirus vector used to immortalize this cell line contains SV40 sequences that can replicate in the presence of BKPyV. In future studies it should be considered the use of a different cell line like the one recently established by Zhao and Imperiale using a lentivirus vector that does not include SV40 sequences (101). Beyond the problems we found with the cell line chosen, when analyzing results obtained using cell lines caution must be taken as they do not always replicate primary cells. Because of this, especially when a strong effect is observed using cell lines, results should be confirmed on primary cells.

PyVAN is attributed not to a primary infection with BKPyV but to a reactivation of a latent infection. As such, it would be interesting to study the drugs' effect in these conditions. A chronic BKPyV infection model was already described on Vero cells (98). Using this model or similar ones, would allow us to test the drugs' efficacy in conditions that are closer to what occurs *in vivo*. Additionally, they could also be used to evaluate whether previous exposure with the drug could be used for prophylaxis, preventing BKPyV reactivation.

Overall, although only a moderate effect was found with these assays for both drugs tested, as this study was only a first approach on screening antiviral drugs against BKPyV, these results can help us to standardize future approaches on evaluating the anti-BKPyV effect of other potentially active drugs. As seen, to get a complete characterization of the drugs' effect, the evaluation of the antiviral effect should be done in the different stages of viral replication to study their temporal effect. Additionally, it is essential to include cytotoxicity assays. These will give us an idea of their toxicity, and together with the results on efficacy will provide information on their selectivity. Another aspect that must always be taken in consideration is the cell type used and the adequacy of the time points chosen in relation to the replication of the virus on those specific cells.

BKPyV reactivation in kidney transplant recipients still poses significant challenges. A better understanding of the virus and host factors that determine BKPyV reactivation and disease outcome could allow a risk stratification of kidney transplant recipients. Continuing the search and development for drugs with antiviral activity against BKPyV is still of great importance.

Defining a standardized high-throughput approach to evaluate antiviral activity would be useful to identify potential drugs with anti-BKPyV activity and compare results between studies. Some drugs have already shown a great anti-BKPyV effect *in vitro* and in some cases their use has already been reported in patients who developed PyVAN after kidney transplantation. Assessing their use in randomized controlled trials is essential so that strong conclusions about their efficacy can be made. Moreover, novel therapeutic approaches are emerging, such as immunotherapy using adoptive T cell transfer. Although their use to treat BKPyV-associated diseases is still in its early stages, further research in this area can bring some promising results. Finally, a vaccine targeting BKPyV that could efficiently prevent BKPyV reactivation in the first place would be a great step on preventing PyVAN development on kidney transplant recipients.

7. References

1. Moens U, Calvignac-Spencer S, Lauber C, Ramqvist T, Feltkamp MCW, Daugherty MD, et al. ICTV virus taxonomy profile: Polyomaviridae. *J Gen Virol*. 2017;98(6):1159–60.
2. Gross L. A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. *Proc Soc Exp Biol Med*. 1953;83(2):414–21.
3. Gardner SD, Field AM, Coleman D V, Hulme B. New human papovavirus (B.K.) isolated from urine after renal transplantation. *Lancet*. 1971;297(7712):1253–7.
4. Padgett BL, Zurhein GM, Walker DL, Eckroade RJ, Dessel BH. Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy. *Lancet*. 1971;297(7712):1257–60.
5. Hirsch HH, Randhawa P, AST Infectious Diseases Community of Practice. BK polyomavirus in solid organ transplantation. *Am J Transplant*. 2013;13(Suppl. 4):179–88.
6. Tan CS, Koralnik IJ. Progressive multifocal leukoencephalopathy and other disorders caused by JC virus: Clinical features and pathogenesis. *Lancet Neurol*. 2010;9(4):425–37.
7. van der Meijden E, Janssens RWA, Lauber C, Bouwes Bavinck JN, Gorbalenya AE, Feltkamp MCW. Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromized patient. *PLoS Pathog*. 2010;6(7):e1001024.
8. Santos-Juanes J, Fernández-Vega I, Fuentes N, Galache C, Coto-Segura P, Vivanco B, et al. Merkel cell carcinoma and Merkel cell polyomavirus: A systematic review and meta-analysis. *Br J Dermatol*. 2015;173(1):42–9.
9. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Malaria and some polyomaviruses (SV40, BK, JC, and Merkel cell viruses). *IARC Monogr Eval Carcinog risks to humans*. 2014;104:9–350.
10. Prado JCM, Monezi TA, Amorim AT, Lino V, Paladino A, Boccardo E. Human polyomaviruses and cancer: an overview. *Clinics (Sao Paulo)*. 2018;73(suppl 1):e558s.
11. Cubitt CL. Molecular genetics of the BK virus. In: *Polyomaviruses and Human Diseases Advances in Experimental Medicine and Biology*, vol 577. New York, NY:

- Springer New York; 2006. p. 85–95.
12. Sweet BH, Hilleman MR. The vacuolating virus, S.V. 40. *Proc Soc Exp Biol Med*. 1960;105:420–7.
 13. Hurdiss DL, Morgan EL, Thompson RF, Prescott EL, Panou MM, Macdonald A, et al. New structural insights into the genome and minor capsid proteins of BK polyomavirus using cryo-electron microscopy. *Structure*. 2016;24(4):528–36.
 14. Ishizu K-I, Watanabe H, Han S-I, Kanesashi S-N, Hoque M, Yajima H, et al. Roles of disulfide linkage and calcium ion-mediated interactions in assembly and disassembly of virus-like particles composed of simian virus 40 VP1 capsid protein. *J Virol*. 2001;75(1):61–72.
 15. Barth H, Solis M, Lepiller Q, Sueur C, Soulier E, Caillard S, et al. 45 years after the discovery of human polyomaviruses BK and JC: Time to speed up the understanding of associated diseases and treatment approaches. *Crit Rev Microbiol*. 2017;43(2):178–95.
 16. Ambalathingal GR, Francis RS, Smyth MJ, Smith C, Khanna R. BK polyomavirus: Clinical aspects, immune regulation, and emerging therapies. *Clin Microbiol Rev*. 2017;30(2):503–28.
 17. Broekema NM, Imperiale MJ. miRNA regulation of BK polyomavirus replication during early infection. *Proc Natl Acad Sci U S A*. 2013;110(20):8200–5.
 18. Pastrana D V., Ray U, Magaldi TG, Schowalter RM, Cuburu N, Buck CB. BK polyomavirus genotypes represent distinct serotypes with distinct entry tropism. *J Virol*. 2013;87(18):10105–13.
 19. Zheng H-Y, Nishimoto Y, Chen Q, Hasegawa M, Zhong S, Ikegaya H, et al. Relationships between BK virus lineages and human populations. *Microbes Infect*. 2007;9(2):204–13.
 20. Nishimoto Y, Zheng H-Y, Zhong S, Ikegaya H, Chen Q, Sugimoto C, et al. An asian origin for subtype IV BK virus based on phylogenetic analysis. *J Mol Evol*. 2007;65(1):103–11.
 21. Yogo Y, Sugimoto C, Zhong S, Homma Y. Evolution of the BK polyomavirus: epidemiological, anthropological and clinical implications. *Rev Med Virol*. 2009;19(4):185–99.
 22. Yogo Y, Zhong S, Xu Y, Zhu M, Chao Y, Sugimoto C, et al. Conserved archetypal configuration of the transcriptional control region during the course of BK polyomavirus

- evolution. *J Gen Virol*. 2008;89(8):1849–56.
23. Gosert R, Rinaldo CH, Funk GA, Egli A, Ramos E, Drachenberg CB, et al. Polyomavirus BK with rearranged noncoding control region emerge in vivo in renal transplant patients and increase viral replication and cytopathology. *J Exp Med*. 2008;205(4):841–52.
 24. Broekema NM, Imperiale MJ. Efficient propagation of archetype BK and JC polyomaviruses. *Virology*. 2012;422(2):235–41.
 25. Hanssen Rinaldo C, Hansen H, Traavik T. Human endothelial cells allow passage of an archetypal BK virus (BKV) strain - A tool for cultivation and functional studies of natural BKV strains. *Arch Virol*. 2005;150(7):1449–58.
 26. Neu U, Allen S-AA, Blaum BS, Liu Y, Frank M, Palma AS, et al. A structure-guided mutation in the major capsid protein retargets BK polyomavirus. *PLoS Pathog*. 2013;9(10):e1003688.
 27. Solis M, Gallais F, Velay A, Fafi-Kremer S. Le virus BK et physiopathologie des maladies associées. *Virologie*. 2019;23(1):7–22.
 28. Jiang M, Abend JR, Tsai B, Imperiale MJ. Early events during BK virus entry and disassembly. *J Virol*. 2009;83(3):1350–8.
 29. Dupzyk A, Tsai B. How polyomaviruses exploit the ERAD machinery to cause infection. *Viruses*. 2016;8(9):242.
 30. Bennett SM, Zhao L, Bosard C, Imperiale MJ. Role of a nuclear localization signal on the minor capsid proteins VP2 and VP3 in BKPyV nuclear entry. *Virology*. 2015;474:110–6.
 31. An P, Sáenz Robles MT, Pipas JM. Large T antigens of polyomaviruses: Amazing molecular machines. *Annu Rev Microbiol*. 2012;66:213–36.
 32. DeCaprio JA, Garcea RL. A cornucopia of human polyomaviruses. *Nat Rev Microbiol*. 2013;11(4):264–76.
 33. Saribas AS, Coric P, Hamazaspyan A, Davis W, Axman R, White MK, et al. Emerging from the unknown: Structural and functional features of agnoprotein of polyomaviruses. *J Cell Physiol*. 2016;231(10):2115–27.
 34. Low J, Humes HD, Szczypka M, Imperiale M. BKV and SV40 infection of human kidney tubular epithelial cells in vitro. *Virology*. 2004;323(2):182–8.
 35. Drachenberg CB, Papadimitriou JC, Wali R, Cubitt CL, Ramos E. BK polyoma virus allograft nephropathy: Ultrastructural features from viral cell entry to lysis. *Am J*

- Transplant. 2003;3(11):1383–92.
36. Henriksen S, Hansen T, Bruun J-A, Rinaldo CH. The presumed polyomavirus viroporin VP4 of simian virus 40 or human BK polyomavirus is not required for viral progeny release. *J Virol*. 2016;90(22):10398–413.
 37. Evans GL, Caller LG, Foster V, Crump CM. Anion homeostasis is important for non-lytic release of BK polyomavirus from infected cells. *Open Biol*. 2015;5(8):150041.
 38. Kean JM, Rao S, Wang M, Garcea RL. Seroepidemiology of human polyomaviruses. *PLoS Pathog*. 2009;5(3):e1000363.
 39. Goudsmit J, Wertheim-van Dillen P, van Strien A, van der Noordaa J. The role of BK virus in acute respiratory tract disease and the presence of BKV DNA in tonsils. *J Med Virol*. 1982;10(2):91–9.
 40. Helle F, Brochot E, Handala L, Martin E, Castelain S, Francois C, et al. Biology of the BKPyV: An update. *Viruses*. 2017;9(11):327.
 41. Doerries K. Human polyomavirus JC and BK persistent infection. In: *Polyomaviruses and Human Diseases Advances in Experimental Medicine and Biology*, vol 577. New York, NY: Springer New York; 2006. p. 102–16.
 42. Egli A, Infanti L, Dumoulin A, Buser A, Samaridis J, Stebler C, et al. Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors. *J Infect Dis*. 2009;199(6):837–46.
 43. Lamarche C, Orio J, Collette S, Sénécal L, Hébert M-J, Renoult É, et al. BK polyomavirus and the transplanted kidney: Immunopathology and therapeutic approaches. *Transplantation*. 2016;100(11):2276–87.
 44. Ramos E, Drachenberg CB, Wali R, Hirsch HH. The decade of polyomavirus BK-A associated nephropathy: state of affairs. *Transplantation*. 2009;87(5):621–30.
 45. Drachenberg CB, Hirsch HH, Ramos E, Papadimitriou JC. Polyomavirus disease in renal transplantation: Review of pathological findings and diagnostic methods. *Hum Pathol*. 2005;36(12):1245–55.
 46. Hirsch HH, Brennan DC, Drachenberg CB, Ginevri F, Gordon J, Limaye AP, et al. Polyomavirus-associated nephropathy in renal transplantation: Interdisciplinary analyses and recommendations. *Transplantation*. 2005;79(10):1277–86.
 47. Borni-Duval C, Caillard S, Olagne J, Perrin P, Braun-Parvez L, Heibel F, et al. Risk factors for BK virus infection in the era of therapeutic drug monitoring. *Transplant J*. 2013;95(12):1498–505.

48. Kuppachi S, Kaur D, Holanda DG, Thomas CP. BK polyoma virus infection and renal disease in non-renal solid organ transplantation. *Clin Kidney J.* 2016;9(2):310–8.
49. Shenagari M, Monfared A, Eghtedari H, Pourkazemi A, Hasandokht T, Khosravi M, et al. BK virus replication in renal transplant recipients: Analysis of potential risk factors may contribute in reactivation. *J Clin Virol.* 2017;96:7–11.
50. Solis M, Velay A, Porcher R, Domingo-Calap P, Soulier E, Joly M, et al. Neutralizing antibody-mediated response and risk of BK virus-associated nephropathy. *J Am Soc Nephrol.* 2018;29(1):326–34.
51. Hirsch HH, Knowles W, Dickenmann M, Passweg J, Klimkait T, Mihatsch MJ, et al. Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. *N Engl J Med.* 2002;347(7):488–96.
52. Hirsch HH, Randhawa PS. BK polyomavirus in solid organ transplantation - Guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. *Clin Transplant.* 2019;e13528.
53. Johnston O, Jaswal D, Gill JS, Doucette S, Fergusson DA, Knoll GA. Treatment of polyomavirus infection in kidney transplant recipients: A systematic review. *Transplantation.* 2010;89(9):1057–70.
54. Bennett SM, Broekema NM, Imperiale MJ. BK polyomavirus: Emerging pathogen. *Microbes Infect.* 2012;14(9):672–83.
55. Hirsch HH, Vincenti F, Friman S, Tuncer M, Citterio F, Wiecek A, et al. Polyomavirus BK replication in *de novo* kidney transplant patients receiving tacrolimus or cyclosporine: A prospective, randomized, multicenter study. *Am J Transplant.* 2013;13(1):136–45.
56. Wali RK, Drachenberg C, Hirsch HH, Papadimitriou J, Nahar A, Mohanlal V, et al. BK virus-associated nephropathy in renal allograft recipients: Rescue therapy by sirolimus-based immunosuppression. *Transplantation.* 2004;78(7):1069–73.
57. Sánchez Fructuoso AI, Calvo N, Perez-Flores I, Valero R, Rodríguez-Sánchez B, García de Viedma D, et al. Mammalian target of rapamycin signal inhibitors could play a role in the treatment of BK polyomavirus nephritis in renal allograft recipients. *Transpl Infect Dis.* 2011;13(6):584–91.
58. Wu JK, Harris MT. Use of leflunomide in the treatment of polyomavirus BK-associated nephropathy. *Ann Pharmacother.* 2008;42(11):1679–85.
59. Teschner S, Gerke P, Geyer M, Wilpert J, Krumme B, Benzing T, et al. Leflunomide

- therapy for polyomavirus-induced Aallograft nephropathy: Efficient BK virus elimination without increased risk of rejection. *Transplant Proc.* 2009;41(6):2533–8.
60. Krisl JC, Taber DJ, Pilch N, Chavin K, Bratton C, Thomas B, et al. Leflunomide efficacy and pharmacodynamics for the treatment of BK viral infection. *Clin J Am Soc Nephrol.* 2012;7(6):1003–9.
61. Chong S, Antoni M, Macdonald A, Reeves M, Harber M, Magee CN. BK virus: Current understanding of pathogenicity and clinical disease in transplantation. *Rev Med Virol.* 2019;29(4):e2044.
62. Blyth E, Clancy L, Simms R, Gaundar S, O'Connell P, Micklethwaite K, et al. BK virus-specific T cells for use in cellular therapy show specificity to multiple antigens and polyfunctional cytokine responses. *Transplantation.* 2011;92(10):1077–84.
63. Pello OM, Innes AJ, Bradshaw A, Finn S-A, Uddin S, Bray E, et al. BKV-specific T cells in the treatment of severe refractory haemorrhagic cystitis after HLA-haploidentical haematopoietic cell transplantation. *Eur J Haematol.* 2017;98(6):632–4.
64. ClinicalTrials.gov. Evaluate tolerability and safety of BD03 for prevention of CMV and BKV reactivation in kidney transplant recipient (Identifier: NCT03576014) [Internet]. [accessed 2019 Apr 10]. Available from: <https://clinicaltrials.gov/ct2/show/study/NCT03576014>
65. Bernhoff E, Tylden GD, Kjerpeseth LJ, Gutteberg TJ, Hirsch HH, Rinaldo CH. Leflunomide inhibition of BK virus replication in renal tubular epithelial cells. *J Virol.* 2010;84(4):2150.
66. Hirsch HH, Yakhontova K, Lu M, Manzetti J. BK polyomavirus replication in renal tubular epithelial cells is inhibited by sirolimus, but activated by tacrolimus through a pathway involving FKBP-12. *Am J Transplant.* 2016;16(3):821–32.
67. Moscarelli L, Caroti L, Antognoli G, Zanazzi M, Di Maria L, Carta P, et al. Everolimus leads to a lower risk of BKV viremia than mycophenolic acid in *de novo* renal transplantation patients: a single-center experience. *Clin Transplant.* 2013;27(4):546–54.
68. Keller F, Wiesner S, Bunjes D, Hartmann B, Schmitt M. Safety and efficacy of everolimus after kidney and hematopoietic stem cell transplantation. *Ann Transplant.* 2012;17(4):52–8.
69. Gois PHF, Rivelli GG, Pereira LM, Mazzali M. Sirolimus in renal transplantation: Analysis of safety and efficacy in a nonprotocol conversion group. *Transplant Proc.* 2012;44(8):2348–51.

70. Bernhoff E, Gutteberg TJ, Sandvik K, Hirsch HH, Rinaldo CH. Cidofovir inhibits polyomavirus BK replication in human renal tubular cells downstream of viral early gene expression. *Am J Transplant*. 2008;8(7):1413–22.
71. Keller LS, Peh CA, Nolan J, Bannister KM, Clarkson AR, Faull RJ. BK transplant nephropathy successfully treated with cidofovir. *Nephrol Dial Transplant*. 2003;18(5):1013–4.
72. Kuypers DRJ, Vandooren A-K, Lerut E, Evenepoel P, Claes K, Snoeck R, et al. Adjuvant low-dose cidofovir therapy for BK polyomavirus interstitial nephritis in renal transplant recipients. *Am J Transplant*. 2005;5(8):1997–2004.
73. Pallet N, Burgard M, Quamouss O, Rabant M, Bererhi L, Martinez F, et al. Cidofovir may be deleterious in BK virus-associated nephropathy. *Transplantation*. 2010;89(12):1542–4.
74. Rinaldo CH, Gosert R, Bernhoff E, Finstad S, Hirsch HH. 1-O-Hexadecyloxypropyl cidofovir (CMX001) effectively inhibits polyomavirus BK replication in primary human renal tubular epithelial cells. *Antimicrob Agents Chemother*. 2010;54(11):4714–22.
75. Papanicolaou GA, Lee YJ, Young JW, Seshan S V., Boruchov AM, Chittick G, et al. Brincidofovir for polyomavirus-associated nephropathy after allogeneic hematopoietics stem cell transplantation. *Am J Kidney Dis*. 2015;65(5):780–4.
76. Reisman L, Habib S, McClure GB, Latiolais LS, Vanchiere JA. Treatment of BK virus-associated nephropathy with CMX001 after kidney transplantation in a young child. *Pediatr Transplant*. 2014;18(7):E227–31.
77. Sharma BN, Li R, Bernhoff E, Gutteberg TJ, Rinaldo CH. Fluoroquinolones inhibit human polyomavirus BK (BKV) replication in primary human kidney cells. *Antiviral Res*. 2011;92(1):115–23.
78. Gabardi S, Waikar SS, Martin S, Roberts K, Chen J, Borgi L, et al. Evaluation of fluoroquinolones for the prevention of BK viremia after renal transplantation. *Clin J Am Soc Nephrol*. 2010;5(7):1298–304.
79. Miller AN, Glode A, Hogan KR, Schaub C, Kramer C, Stuart RK, et al. Efficacy and safety of ciprofloxacin for prophylaxis of polyomavirus BK virus-associated hemorrhagic cystitis in allogeneic hematopoietic stem cell transplantation recipients. *Biol Blood Marrow Transplant*. 2011;17(8):1176–81.
80. Knoll GA, Humar A, Fergusson D, Johnston O, House AA, Kim SJ, et al. Levofloxacin for BK virus prophylaxis following kidney transplantation. *JAMA*. 2014;312(20):2106.

81. Randhawa P, Pastrana D V., Zeng G, Huang Y, Shapiro R, Sood P, et al. Commercially available immunoglobulins contain virus neutralizing antibodies against all major genotypes of polyomavirus BK. *Am J Transplant.* 2015;15(4):1014–20.
82. Sharma AP, Moussa M, Casier S, Rehman F, Filler G, Grimmer J. Intravenous immunoglobulin as rescue therapy for BK virus nephropathy. *Pediatr Transplant.* 2009;13(1):123–9.
83. Vu D, Shah T, Ansari J, Naraghi R, Min D. Efficacy of intravenous immunoglobulin in the treatment of persistent BK viremia and BK virus nephropathy in renal transplant recipients. *Transplant Proc.* 2015;47(2):394–8.
84. Icheva V, Kayser S, Wolff D, Tuve S, Kyzirakos C, Bethge W, et al. Adoptive transfer of Epstein-Barr virus (EBV) nuclear antigen 1-specific T cells as treatment for EBV reactivation and lymphoproliferative disorders after allogeneic stem-cell transplantation. *J Clin Oncol.* 2013;31(1):39–48.
85. Crough T, Beagley L, Smith C, Jones L, Walker DG, Khanna R. *Ex vivo* functional analysis, expansion and adoptive transfer of cytomegalovirus-specific T-cells in patients with glioblastoma multiforme. *Immunol Cell Biol.* 2012;90(9):872–80.
86. Papadopoulou A, Gerdemann U, Katari UL, Tzannou I, Liu H, Martinez C, et al. Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. *Sci Transl Med.* 2014;6(242):242ra83.
87. Wieser M, Stadler G, Jennings P, Streubel B, Pfaller W, Ambros P, et al. hTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics. *Am J Physiol Renal Physiol.* 2008;295(5):F1365-75.
88. Bennett SM, Jiang M, Imperiale MJ. Role of cell-type-specific endoplasmic reticulum-associated degradation in polyomavirus trafficking. *J Virol.* 2013;87(16):8843–52.
89. Marshall WF, Telenti A, Proper J, Aksamit AJ, Smith TF. Rapid detection of polyomavirus BK by a shell vial cell culture assay. *J Clin Microbiol.* 1990;28(7):1613–5.
90. Jeffers LK, Madden V, Webster-Cyriaque J. BK virus has tropism for human salivary gland cells in vitro: Implications for transmission. *Virology.* 2009;394(2):183–93.
91. Barth H, Solis M, Kack-Kack W, Soulier E, Velay A, Fafi-Kremer S. *In vitro* and *in vivo* models for the study of human polyomavirus infection. *Viruses.* 2016;8(10).
92. Sundsfjord A, Johansen T, Flaegstad T, Moens U, Villand P, Subramani S, et al. At least two types of control regions can be found among naturally occurring BK virus

- strains. J Virol. 1990;64(8):3864.
93. Teunissen EA, de Raad M, Mastrobattista E. Production and biomedical applications of virus-like particles derived from polyomaviruses. J Control Release. 2013;172(1):305–21.
 94. Li T-C, Takeda N, Kato K, Nilsson J, Xing L, Haag L, et al. Characterization of self-assembled virus-like particles of human polyomavirus BK generated by recombinant baculoviruses. Virology. 2003;311(1):115–24.
 95. Mahon C, Liang B, Tikhanovich I, Abend JR, Imperiale MJ, Nasheuer HP, et al. Restriction of human polyomavirus BK virus DNA replication in murine cells and extracts. J Virol. 2009;83(11):5708–17.
 96. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Epidemiol. 1938;27(3):493–7.
 97. Handala L, Fiore T, Rouillé Y, Helle F. QuantIF: An ImageJ macro to automatically determine the percentage of infected cells after immunofluorescence. Viruses. 2019;11(2):165.
 98. Acott PD, O'Regan PA, Lee SH, Crocker JF. Utilization of Vero cells for primary and chronic BK virus infection. Transplant Proc. 2006;38(10):3502–5.
 99. Li R, Sharma BN, Linder S, Gutteberg TJ, Hirsch HH, Rinaldo CH. Characteristics of polyomavirus BK (BKPyV) infection in primary human urothelial cells. Virology. 2013;440(1):41–50.
 100. Tylden GD, Hirsch HH, Rinaldo CH. Brincidofovir (CMX001) inhibits BK polyomavirus replication in primary human urothelial cells. Antimicrob Agents Chemother. 2015;59(6):3306–16.
 101. Zhao L, Imperiale MJ. Establishing renal proximal tubule epithelial-derived cell lines expressing human telomerase reverse transcriptase for studying BK polyomavirus. Newton ILG, editor. Microbiol Resour Announc. 2019;8(42).
 102. Funk GA, Gosert R, Comoli P, Ginevri F, Hirsch HH. Polyomavirus BK replication dynamics *in vivo* and *in silico* to predict cytopathology and viral clearance in kidney transplants. Am J Transplant. 2008;8(11):2368–77.
 103. Edwards TG, Fisher C. Antiviral activity of pyrrole-imidazole polyamides against SV40 and BK polyomaviruses. Antiviral Res. 2018;152:68–75.